Lignocellulose degradation and humus modification by the fungus *Paecilomyces inflatus*

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Academic Dissertation in Microbiology

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Cover photo: Microscopic picture of *Paecilomyces variotti*. (photo by David Ellis, University of Adelaide, Australia)

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List of original publications

The thesis is based on the following publications referred to in the text by Roman numerals I- IV. In addition, unpublished data are also presented.

I Kluczek-Turpeinen B., Tuomela M., Hatakka A. and Hofrichter M. 2003 Lignin degradation in a compost environment by the deuteromycete *Paecilomyces inflatus*. Applied Microbiology and Biotechnology 61: 374-379

II Kluczek-Turpeinen B., Steffen K.T., Tuomela M., Hatakka A. and Hofrichter M. 2005 Modification of humic acids by the compost-dwelling deuteromycete *Paecilomyces inflatus*. Applied Microbiology and Biotechnology 66: 443-449

III Kluczek-Turpeinen B., Maijala P., Tuomela M., Hofrichter M. and Hatakka A. 2005 Endoglucanase activity of compost-dwelling fungus *Paecilomyces inflatus* is stimulated by humic acids and other low molecular mass aromatics**.** World Journal of Microbiology and Biotechnology 21: 1603-1609

IV Kluczek-Turpeinen B., Maijala P., Hofrichter M. and Hatakka A. 2007 Degradation and enzymatic activities of three *Paecilomyces inflatus* strains grown on diverse lignocellulosic substrates. International Biodeterioration and Biodegradation 59: 283-291

The author´s contribution

I Beata Kluczek-Turpeinen planned the experiments, did the laboratory work. She interpreted the results and wrote the paper.

II Beata Kluczek-Turpeinen planned the experiments, did the laboratory work except the HPSEC analyses. She interpreted the results and wrote the paper.

III Beata Kluczek-Turpeinen planned the experiments, did the laboratory work, analyzed the data and wrote the paper together with Pekka Maijala.

IV Beata Kluczek-Turpeinen planned the experiments, did the laboratory work. She interpreted the results and wrote the paper. Pekka Maijala supervised part of the practical work and took part in the interpretation of the results and writing the paper.

Abbreviations

ABTS	2,2`-azinobis(3-ethylbenzthiazoline-6-sulphonate)
BGL	*β*-glucosidases
CDH	cellobiohydrolase
CM-cellulose	carboxymethyl-cellulose
DHP	dehydrogenation polymer (synthetic lignin)
EG	endoglucanase
FA	fulvic acid
FPA	filter paper assay
G	guaiacyl
GM	composted grape marc
HA	humic acid
HBT	hydroxybenzotriazole
HPSEC	high performance size exclusion chromatography
HS	humic substances
ITS	internal transcribed spacer
kDa	kiloDalton
LiP	lignin peroxidase
MnP	manganese peroxidase
MSW	municipal solid waste
MW	molecular weight
MM	molecular mass
p*I*	isoelectric point
RNA	ribonucleic acid
S	syringyl
SHA	soil humic acid
S	sewage sludge
SSC	solid-state cultivation
VP	versatile peroxidases
WC	wood compost

Abstract

Composting is the biological conversion of solid organic waste into usable end products such as fertilizers, substrates for mushroom production and biogas. Although composts are highly variable in their bulk composition, composting material is generally based on lignocellulose compounds derived from agricultural, forestry, fruit and vegetable processing, household and municipal wastes. Lignocellulose is very recalcitrant; however it is rich and abundant source of carbon and energy. Therefore lignocellulose degradation is essential for maintaining the global carbon cycle. In compost, the active component involved in the biodegradation and conversion processes is the resident microbial population, among which microfungi play a very important role. In composting pile the warm, humid, and aerobic environment provides the optimal conditions for their development. Microfungi use many carbon sources, including lignocellulosic polymers and can survive in extreme conditions. Typically microfungi are responsible for compost maturation.

In order to improve the composting process, more information is needed about the microbial degradation process. Better knowledge on the lignocellulose degradation by microfungi could be used to optimize the composting process. Thus, this thesis focused on lignocellulose and humic compounds degradation by a microfungus *Paecilomyces inflatus*, which belongs to a flora of common microbial compost, soil and decaying plant remains. It is a very common species in Europe, North America and Asia. The lignocellulose and humic compounds degradation was studied using several methods including measurements of carbon release from ^{14}C-labelled compounds, such as synthetic lignin (dehydrogenative polymer, DHP) and humic acids, as well as by determination of fibre composition using chemical detergents and sulphuric acid. Spectrophotometric enzyme assays were conducted to detect extracellular lignocellulose-degrading hydrolytic and oxidative enzymes.

Paecilomyces inflatus secreted clearly extracellular laccase to the culture media. Laccase was involved in the degradation process of lignin and humic acids. In compost *P. inflatus* mineralised 6–10% of ^{14}C-labelled DHP into carbon dioxide. About 15% of labelled DHP was converted into water-soluble compounds. Also humic acids were partly mineralised and converted into water-soluble material, such as low-molecular mass fulvic acid-like compounds. Although laccase activity in aromatics-rich compost media clearly is connected with the degradation process of lignin and lignin-like compounds, it may preferentially effect the polymerisation and/or detoxification of such aromatic compounds. *P. inflatus* can degrade lignin and carbohydrates also while growing in straw and in wood. The cellulolytic enzyme system includes endoglucanase and β-glucosidase. In *P. inflatus* the secretion of these enzymes was stimulated by low-molecular-weight aromatics, such as soil humic acid and veratric acid. When strains of *P. inflatus* from different ecophysiological origins were compared, indications were found that specific adaptation strategies needed for lignocellulosics degradation may operate in *P. inflatus*. The degradative features of these microfungi are on relevance for lignocellulose decomposition in nature, especially in soil and compost environments, where basidiomycetes are not established. The results of this study may help to understand, control and better design the process of plant polymer conversion in compost environment,with a special emphasis on the role of ubiquitous microfungi.

Tiivistelmä (Abstract in Finnish)

Kompostoitumisella tarkoitetaan kiinteän orgaanisen aineen biologista muuntumista hyödynnettäviksi lopputuotteiksi, kuten vaikkapa lannoitteiksi, ruokasienten kasvatusalustoiksi sekä biokaasuksi. Kompostien koostumus vaihtelee suuresti; kuitenkin useimmiten kompostin perustana ovat kasviperäiset materiaalit sekä talousjätteet. Kasviaines on pääosin rakentunut puuaineesta eli ligniinistä sekä selluloosasta. Kasviainekseen on sitoutunut valtava määrä hiiltä ja energiaa. Kokonaisuutena lignoselluloosan hajotus on välttämätöntä maapallon hiilen kierron kannalta.

Kompostissa kompostin mikrobit, erityisesti mikrosienet, muuntavat ja hajottavat lignoselluloosaa. Kompostin lämmin, kostea ilma takaa mikrosienille ihanteelliset olot sienten kasvuun ja kasviaineksen hajotukseen. Monet mikrosienet ovat sopeutuneet selviytymään äärimmäisissäkin olosuhteissa.

Kasviaineksen kompostoitumistehokkuutta voidaan lisätä, jos kompostoitumisen mikrobiologia ja erityisesti lignoselluloosan hajotus tunnetaan hyvin. Tämän väitöskirjatyön tarkoituksena oli tutkia kompostissa elävän *Paecilomyces inflatus* –mikrosienen lignoselluloosan sekä humusyhdisteiden hajotusta. Sieni on sangen tavallinen maaperän ja kompostien sieni sekä Euroopassa, Aasiassa että Pohjois-Amerikassa. Sienen on todettu kykenevän kasvamaan myös puussa. Lignoselluloosan ja humusyhdisteiden hajotusta tutkittiin usein eri menetelmin, mm. käyttämällä radioaktiivisella hiilellä leimattuja malliyhdisteitä, analysoimalla kasviaineksen puuaineen määrää sekä tutkimalla sienen puuainetta hajottavien entsyymien erittymistä kasvualustaan.

Tutkimuksissa selvisi että kompostista eristetty mikrosieni *P. inflatus* osoitti selvästi solunulkoisten hapettavien entsyymien kuten lakkaasin tuottoa. Lakkaasin avulla sieni pystyi hajottamaan jonkin verran puuainetta ja humusyhdisteitä, kuten humushappoja, joiden tärkein lähtöaine on puuaine eli ligniini.

Kompostissa mikrosieni hajotti 6–10% radioaktiivisella hiilellä leimatusta puuaineesta hiilidioksidiksi. Leimautuneita vesiliukoisia yhdisteitä muodostui 15%. Sieni tuotti humushaposta hiilidioksidia ja pienimolekyylisiä yhdisteitä kompostiviljemässä. Paitsi kompostissa *P. inflatus* hajottaa puuainetta ja hiilihydraatteja myös olkialustalla ja puussa. Sieni näyttää olevan hyvin sopeutunut myös näihin olosuhteisiin. Selluloosaa hajottavista entsyymeistä sieni tuotti endoglukanaasia ja β-glukosidaasia.

Tulokset osoittavat, että näillä sienillä voi olla merkittävä osuus ligniinin, humuksen ja lignoselluloosan hajotuksessa niin kompostissa kuin maassakin, eli ympäristöissä, missä varsinaisten puuta lahottavien kantasienten elinkyky on rajoittunut. Tutkimustyön tulokset auttavat paremmin ymmärtämään, hallitsemaan ja suunnittelemaan kasviaineksen kompostihajotusta.

Tulokset vahvistavat mikrosienten keskeistä osuutta osana hyvän kompostin toimintaa.

1. INTRODUCTION

1.1. Microfungi

1.1.1. Characteristics and importance of microfungi

Microfungi are diverse group of fungi consisting of yeasts and molds (Gravesen et al. 1994). From the taxonomic point of view, most microfungi belong to the Ascomycetes, mitosporic fungi (Deuteromycetes) and Zygomycetes. About 29 000 ascomycetous species are known so far. Mitosporic fungi, formerly designated as Deuteromycetes, account for about 17 000 species. The Zygomycetes are unimpressive in numbers of species, approximately only 700 being known (Gow and Gadd 1996). The fungal body consists of microscopic threads called hyphae, extending through the substrate through which they grow. Typically only the "fruiting body" of the fungus is visible, producing thousands of tiny spores that are carried by the air, spreading the fungus to new locations. Spores are produced in a variety of ways and occur in a bewildering array of shapes and sizes. In spite of this diversity, spores are quite constant in their shapes, sizes (about 2–20 μm), colour and form. Thus these characteristics are very useful for identification of microfungi. The most basic difference between spores lies in their method of initiation, which can be either sexual or asexual (Carlile et al. 2001).

Microfungi are well adapted to extreme environmental conditions. They tolerate a wide range of temperature, pH, dryness, oxygen concentrations and ultraviolet radiation better than the wood-rotting basidiomycetes called white or brown rot fungi. In addition they are found in all climatic zones ranging from the poles to the tropics (Blanchette 2000, Blanchette et al. 2004). Generally, fungi prefer an acidic environment (Deacon 1997) although microfungal activities occur within a board pH range of between 3.7 and 8.6 (Daniel and Nilsson 1998). Microfungi are found in acid coniferous forest soils in addition to neutral soils and composts (Tuomela et al. 2000, Daniel and Nilsson 1998) and they can successfully colonize exposed aerial surfaces, in conditions, which may be preventative to the growth of other microorganisms (Carlile et al. 2001, Blanchette 2000). Moreover, microfungi can protect themselves by relatively quick growth in natural niches and by the production of antibiotics and toxic substances (mycotoxins). They can also serve as feed for insects and as symbiotic partners with algae and cyanobacteria in lichens (Gravesen et al. 1994).

Microfungi are common saprophytes that exist in: soil *Trichoderma*, *Penicillium*; (Domsch et al.1980), compost *Chaetomium (*Chefetz et al. 1998), wood *Xylaria* and *Hypoxylon* (Pointing et al. 2003), and in water environment *Ophioceras dolichostomum*, *Savoryella lignicola* (Bucher et al. 2004). Many microfungi are important plant pathogens, including *Ophiostoma novo-ulmi* in Dutch elm disease (Gow and Gadd 1996), *Claviceps purpurea* causing ergot of cereals (Gravesen et al. 1994)) and *Fusarium solani* f. sp. *glycines* that cause root rots of soybean (Lozoyova et al. 2006). Some other microfungi are parasities of insects e.g. *Beauveria* or nematodes e.g. *Arthrobotrys* (Gow and Gadd 1996).

In soil, microfungi usually exist in the organic upper layers (humus and topsoil), although some species have also been found in underlying rocky layers (subsoil) (Dix and Webster 1995). Similarly in compost they mainly occupy the upper parts of compost (>10 cm depth) where oxygen remains available (Millner et al. 1977). In both habitats microfungi together with bacteria, other fungi and animals participate in the decomposition of organic matter to carbon dioxide and humus (Dix and Webster 1995).

Microfungi are able to colonize and cause soft-rot decay of wood (Daniel and Nilsson 1998). Soft rot decomposition can even occur in wood with high content of tannins and other compounds normally resistant to microbial attack. In addition soft rot is associated with hot, wet and cold conditions, which inhibits colonization by the more aggressive white and brown rotting fungi (Blanchette 2000, Blanchette et al. 2004). The wood decayed by soft rot has a brown soft appearance that is cracked and checked when dry (Blanchette 1995). Microfungi preferably colonize and degrade hardwood. In softwood the rate of the wood decay by microfungi is generally lower than in hardwood (Kuhad et al. 1997). Weight losses of up to 50 % in birch wood (hardwood) and 20 % in pine wood (softwood) within 3 months caused by these fungi have been reported (Nilsson et al. 1989, Ferraz and Duran 1995). Microfungi preferentially metabolize wood polysaccharides and produce an array of cellulolytic and hemicellulolytic activities that may contribute to the degradation of plant cell wall material (Kubicek and Penttilä 1998, de Vries and Visser 2001, Tribak et al. 2002). They are also capable of some direct transformation of lignin from the outer layers of the cell walls, however they leave the middle lamella intact (Blanchette 1995). On the other hand , many microfungi have been reported to degrade synthetic lignin to CO_2 and water-soluble products (Haider and Trojanowski 1975, Rodriguez et al. 1996b, Regalado et al. 1997, Gonzalez et al. 2002, Liers et al. 2006) and rapidly convert lignin-related phenolic compounds (Ander et al. 1984, Betts and Dart 1988, Bugos et al. 1988, Hofrichter et al. 1993, Hofrichter et al. 1994, Leitão et al. 2007). Such abilities of microfungi may be linked to their capability for lignin degradation. Table 1 presents the spectrum of microfungi species involved in the production of various lignocellulolytic enzymes in solid state cultivation systems.

In addition to the important role of microfungi in carbon cycling, they are also involved in many biotechnological processes. These processes include: brewing, winemaking, baking, cheese making and the preparation of other fermented food (e.g. tempe, miso, angkak, soy sauce) together with edible mushroom production are the most important microfungal applications. Production of enzymes (amylase, cellulase, invertase, lipase, pectinase, proteinase, rennin and xylanase), organic acids (citric, itaconic and lactic acids), antibiotics and other pharmaceuticals (penicillin, mevinolin, cephalosporin, griseofulvin and cyclosporine) by fungi are common processes that have been reviewed (Bennet 1998 and Demain 1999).

1.1.2. Microfungi in compost

1.1.2.1. Composting environment

The degradation of organic wastes is a natural process and begins almost as soon as the wastes are generated. Composting is a means of controlling and accelerating the decomposition process. This involves the self-heating and aerobic biological breakdown of or-

Table 1. Microfungal species used for the production of various lignocellulolytic enzymes in solid state cultivation systems

Substrate	Microfungus	Enzyme	Reference
Wheat bran, wheat straw, banana leaf waste	*Aspergillus* sp., *A. terreus, A. niger*	CM-cellulase, FPA, β-glucosidase, CBH, xylanase, laccase, LiP	Ghanem et al. 2000, Hanif et al. 2004, Shah et al. 2005
Softwood Kraft lignin	*Botryosphaeria* sp.	laccase	Dekker et al. 2001
Wheat straw	*Botrytis cinerea*	cellulase, xylanase	Thygesen et al. 2003
Wheat bran, sugar beet pulp, wheat straw, palm fruit fibre	*Chaetomium globosum*	xylanase, cellulases	Wiacek-Zychlinska et al. 1994, Umikalsom et al. 1998
Wheat straw, compost	*Chaetomium thermophilum*	xylanase, laccase	Latif et al. 2006, Chefetz et al. 1998,
Bagasse	*Humicola grisea var. thermoidea*	CBH, FPA, β-glucosidase, xylanase	De-Paula et al. 1999, Salles et al. 2005
Bagasse, wheat straw, rice straw, rice husks, barley bran	*Melanocarpus* sp., *M. albomyces*	xylanases, endoglucanase, CBH	Prahbu and Meheshwari 1999, Saraswat and Bisaria 2000, Jatinder et al. 2006, Kaur et al. 2006
Bagasse, corn cob, rice straw, wheat straw, wheat bran	*Myceliophthora* sp.	cellulases, xylanases	Badhan et al. 2007
Wheat straw	*Neurospora crassa*	endoglucanase	Romero et al. 1999
Wheat straw	*Paecilomyces thermophila*	xylanase	Yang et al. 2006
Rice straw, Corn cob, oat husk, agricultural residues	*Penicillium* sp., *P. simplicissimum, P. janthinellum, P. brasilianum*	laccase, cellulases xylanases	Rahman et al. 2003, Thygesen et al. 2003, Oliveira et al. 2006 Zeng et al. 2006

Bagasse, grass clippings, rice straw	*Scytalidium thermophilum*	endoglucanase, exoglucanase, β-glucosidase,	Ögel et al. 2001, Kaur et al. 2006
Wheat straw	*Sporotrichum thermophile*	xylanase	Topakas et al. 2003
Wheat straw, wheat bran, baggase, agricultural residues	*Thermoascus auranticus*	endoglucanase, xylanase, phenol oxidase	Machuca et al. 1998, Kalogeris et al. 2003, Milagres et al. 2003
Corncob	*Thermomyces lanuginosus*	xylanase	Damaso et al. 2000
Wheat straw	*Trichoderma longibrachiatum*	CM-cellulase, β-glucosidase, laccase, MnP	Velazquez -Cedeño et al. 2004
Birchwood	*Xylaria polymorpha**	endoglucanase, β-glucosidase, esterase, xylanase, laccase	Liers et al. 2006

ganic materials, with successions of different microbes, in which temperature, pH and availability of nutrients constantly change (Biddlestone and Gray 1985, Epstain 1997, Tuomela et al. 2000, Ryckeboer et al. 2003b). A scheme of composting process in shown in Figure 1. Composting may mineralize the simpler and more easily assimilated compounds and humify complex substrates into usable end products such as fertilizers, substrates for mushroom production and biogas (Crawford 1983, Epstain 1997). Although composts are highly variable in bulk composition, they are generally based on lignocellulose compounds, together with other substrates derived from agricultural, forestry, fruit and vegetable processing as well as household and municipal wastes.

Successful composting depends on a number of optimal factors including : an adequate supply of oxygen, correct particle size, moisture, C/N ratio and pH. These factors influence the type of microorganisms, species diversity and the rate of decomposition (Crawford 1983). The key parameters of composting are given in Table 2. The complexity of degraded plant materials and the quality of the final product may depend upon the type of waste (Biddlestone and Gray 1985).

The resident microbial community in compost consists of bacteria, actinomycetes and fungi, Resident microbial communities have recently been reviewed by Tuomela et al. (2000). During the various composting phases different microbial communities predominate, each of which is adapted to the particular environment (Ryckeboer et al. 2003 a, b Table 3). At the beginning of composting mesophilic bacteria predominate, but when the temperature increases to over 40°C, thermophilic bacteria and fungi predominate in the compost. Temperatures of over 60°C are critical for microorganisms thus microbial activity decreases dramatically but after the compost has cooled mesophilic bacteria and actinomycetes again predominate (Ryckeboer et al. 2003 b). Among micro-

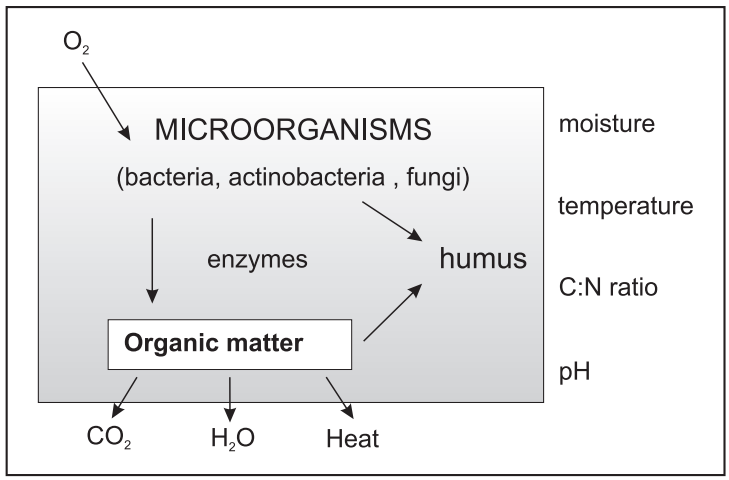

Figure 1. The composting process and important compost factors affecting this process adapted from Itävaara et al. (1995)

bial organisms, microfungi play a very important role. They can use many carbon sources including lignocellulose polymers and they can survive in variable conditions. Therefore, microfungi are mainly responsible for compost maturation (Maheshwari et al. 2000, Tuomela et al. 2001) and compost seems to be an excellent habitat for them since it contains all organic substrates necessary for microbial growth and reproduction.

During the composting process temperature, pH and nutrient availability constantly change therefore these factors influence the types of microorganisms, species diversity and the rate of metabolic activities. Degradation of waste materials in compost proceeds in three phases: (i) the mesophilic phase, (ii) the thermophilic phase and (iii) the cooling and maturation phase, all of which differ in temperature, pH values and microbial consortia (Figure 2).

Table 2. Composting parameters (Biddlestone and Gray 1985)

Major parameters	Optimum value
Nutrient balance (C:N ratio)	35:1
Water content	50–75 % depends on material
Particle size	12.5 mm for agitated plants and forced aeration 50 mm for windrows and natural aeration
Air flow	0.6-1.8 m^3 air d^{-1} kg^{-1} volatile solids during thermophilic phase, being progressively decreased during cooling down and maturing
pH	6.5–8.0
Oxygen concentration	> 10 %
Temperature	55 °C span 50–65°C

Table 3. Species diversity of the dominant microorganisms isolated during different composting phases (van Heerden et al. 2002 and Ryckeboer et al. 2003a).

Composting phase	Prokaryotes	Fungi
Mesophilic phase	*Bacillus macerans*	*Aspergillus flavus*
	Staphylococcus saprophiticus	*Aspergillus niger*
	Flavobacterium sp.	*Aspergillus ustus*
	Streptomyces sp.	*Penicillium sublateritum*
	Rhodococcus rhodochrous	*Eupenicillium cinnamopurpureum*
	Micrococcus	*Cladosporium cladosporioides*
	Nocardia otitidiscaviarium	
Thermophilic phase	*Enterobacter cloacae*	*Absidia corymbifera*
	Coryneform sp.	*Penicillium diversum*
	Paenibacillus macerans	*Paecilomyces variotii*
	Bacillus licheniformis	*Rhizomucor pusillus*
	Staphylococcus capitis	*Thermomyces lanuginosus*
	Brevibacillus agri	*Thermomyces. ibananensis*
Cooling and maturation phase	*Alcaligenes denitrificans*	*Fusarium solani*
	Proteus vulgaris	*Paecilomyces lilacinus*
	Pseudomonas aeruginosa	*Coprinus lagopus*
	Serratia marcescens	*Mucor sp.*
	Cellulomonas cellulans	*Thrichothecium sp.*
	Bacillus sphaericus	*Geotrichum candidum*
	Flavobacterium mizutaii	*Memnoniella echinata*

1.1.2.2. Occurrence and role of microfungi in compost

Microfungi are the main components of the microflora that develop in heaped masses of plant material and piles of agricultural and forestry products wherein a warm, humid and aerobic environment provides the best conditions for their development. They grow in compost in all phases but may disappear temporarily during peak heating (Trambirajah et al. 1995). Microfungi constitute a heterogeneous physiological group of various genera in the Ascomycetes, Zygomycetes and mitosporic fungi formarly know as Deuteromycetes (Maheshwari et al. 2000).

Van Heerden et al. (2002) and Ryckeboer et al. (2003a) followed the succession of microfungi in a compost environment. They found a freshly made compost heap contains a variety of soil and leaf-inhabiting fungal genera. The majority of these fungi are mesophiles with maximum growth temperatures between 25 and 30°C whereas other genera are thermotolerant and are capable of growth over the range of 40 to 50°C. At temperature above 60°C is the upper limit of growth for fungi in addition to all other

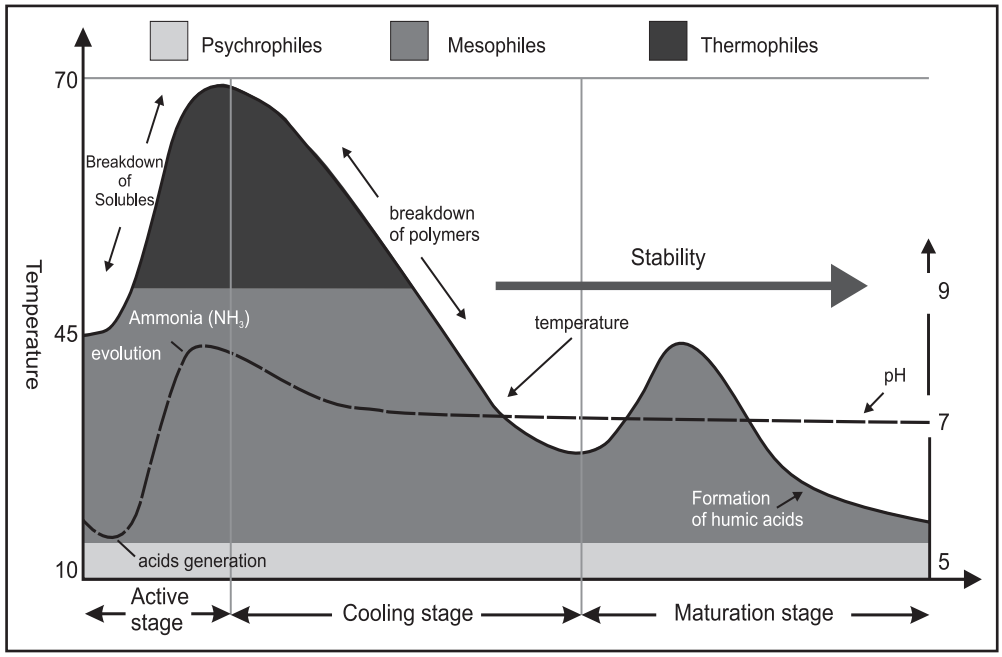

Figure 2. Composting phases modified from Biddlestone and Gray (1985)

eukaryotes (Kane and Mullins 1973). In some studies the raw material of compost has been found to contain approximately 10^6 microbial counts of mesophilic fungi per gram of raw material and *Aspergillus* and *Penicillium* are the predominant fungal genera (von Klopotek 1962, Trambirajah et al. 1995, van Heerden et al. 2002). As the pile temperature increases to the thermophilic range, thenumber of fungi rises and they efficiently inhabit the pile. However, the counts of the fungi decreases to 10^3 per g of compost as the temperature rises to above 60°C and at 64°C all these fungi disappear (von Klopotek 1962, Trambirajah et al. 1995). Interestingly, the mesophilic fungus *Cladosporium cladosporioides* was able to grow over the 64 to 65°C range (von Klopotek 1962). The fungi survive at high temperatures which is most likely due to the short duration of the exposure to the high temperatures (Trambirajah et al. 1995). Temperature tolerance also differs within genera and even within the fungal species. The growth substrate may also have some influence on temperature tolerance (Ofosu-Asiedu and Smith 1973a). *Sporotrichum thermophile* Apinis (syn. *Myceliophthora thermophila*) produces extracellular cellulases on sugarcane bagasse even at 65°C (El-Naghy et al. 1991), whereas *Talaromyces emersonii* is still active and can grow after four weeks at elevated temperatures (Ofosu-Asiedu and Smith 1973a). As the temperature in compost falls below 60°C, both mesophilic and thermophilic microfungi start to re-colonise the substrate (von Klopotek 1962, Trambirajah et al. 1995). Among the mesophilic fungi a few lignin-degrading Basidiomycota including *Coprinus* sp., *Panaeolus* sp., *Corticium coronilla, Trametes* sp. and *Phanerochaete* sp. have been isolated from compost at the cooling and maturation phases or from mature compost (von Klopotek 1962, Granit et al. 2007). The basidiomycete *Coprinus cinereus* is an example of a degrader of polymers, otherwise resistant to degradation. This fungus has a maximum growth temperature of about 40°C and prefers an alkaline environment (Dix and Webster 1995, Deacon 1997).

Most microfungi are obligate aerobes consequently they have a lower tolerance for low oxygen partial pressure than bacteria. For this reason, microfungi mostly live in the outer layer of compost where they grow both as unseen filaments and fuzzy grey or white colonies on the compost surface. Thus, oxygen can be a limiting factor not only for the growth but also for their metabolic activity (Walsh 1972). Interestingly, oxygen deficiency brings about morphogenetic changes in *Talaromyces* (*Penicillium*) *duponti* (Cooney and Emerson 1964). The fungus forms only a conidial stage (*Penicillium*) in aerobic cultures. The sexual stage (*Talaromyces*) is initiated in agar cultures only when they are flushed with nitrogen.

Compost microfungi are less sensitive to environments with low moisture and pH than bacteria. Therefore microfungi can attack organic residues that are too dry and acidic or too low in nitrogen for bacterial decomposition. Nitrogen addition often increases the rate of lignin attack by most microfungi, in contrast to that found for the basidiomycetous white rotting fungi (Daniel and Nilsson 1998). Nitrogen availability is often also the limiting factor for cellulose degradation (Dix and Webster 1995).

Both thermophile and mesophile microfungi are responsible for the decomposition of many complex plant polymers during composting. They break down otherwise recalcitrant debris, enabling bacteria to continue further the decomposition process after most of the cellulose has been exhausted. A range of cellulolytic microfungi colonize after peak-heating, then grow over the next 10 to 20 days. They rapidly decompose cellulose in compost, but enzyme activity of the respective culture filtrates has been found to be low when compared to that of the mesophilic fungus *Hypocrea jecorina* (anamorph *Trichoderma reesei*) (Bhat and Maheshwari et al. 1987). Interestingly, some compost fungi are unable to utilize cellulose for example *Thermomyces lanuginosus,* but this fungi can grow commensally by utilizing sugars generated by other fungi and perhaps also by using their mycelial breakdown products (Puchart et al. 1999). Moreover, several noncellulolytic species readily utilize xylan, the major hemicellulose component of the cell walls of many plants (Prabhu and Maheshwari 1999). The ability of microfungi to hydrolyze hemicellulose is probably more common than cellulose cleavage (Dix and Webster 1995). During the prolonged phase of warm temperature that follows peak-heating, a compost can lose up to 50 % of its dry weight. This loss comprises nearly two-thirds of the main plant cell wall components including cellulose and hemicellulose (Deacon 1997).

Lignin biodegradation is an important activity during composting because of its involvement in humification and release of nutrients to microorganisms. Tuomela et al. (2001) found a rather high mineralization of ^{14}C-labelled synthetic lignin (^{14}C-DHP) preparation by mixed microbial population in a compost environment. A noticeably higher degradation occurred at 35˚C and 50˚C (23–24 %) than at 58˚C (7 %). This points to an involvement of eukaryotic organisms (very probably microfungi) in the degradation process, since their activity is strongly suppressed at temperatures at 58ºC and above. Waksman et al. (1939) examined the lignin degradation capacity of some microorganisms isolated from compost, and found that the thermophilic ascomycete *Thermomyces lanuginosus* degraded 4.2 % of lignin at 50°C over 42 days. *Thermoascus aurantiacus* degraded 15 % of wood lignin in a 21 day cultivation period (Machuca and Duran 1993). *Aspergillus* spp. has a high lignin-degrading capacity (Shah et al. 2005). Some of them have been isolated from compost (von Klopotek 1962, Van Heerden et al. 2002).

Low lignin-degrading activities have also been found for *Paecilomyces* spp., *Thielavia terrestris* and *Talaromyces thermophilus* (Eslyn et al. 1975, Dix and Webster 1995). *Melanocarpus albomyces* a thermophilic ascomycete that commonly grows in the hottest parts of the compost (Prahbu and Maheshwari 1999) produces laccase that is able to bind to cellulose (Kiiskinen et al. 2004). Compost microfungi, which are known to have lignocellulolytic activity or which grow on lignocellulose or compost, are listed in Table 4.

1.1.3. *Paecilomyces inflatus*

The genus *Paecilomyces* includes 31 species divided into two sections *Paecilomyces* and *Isarioidea* (Samson 1974). The classification is based on their morphological characteristics. The section *Paecilomyces* contains members that are often thermophilic whereas *Isarioidea* contains mesophiles, including several entomopathogenic or nematophagous species. However, data obtained from molecular studies modify the systematics of these fungi. Comparative analyses of ribosomal RNA gene sequences and internal transcribed spacer (ITS) sequences indicate the polyphyletic character of the genus *Paecilomyces* (Obornik et al. 2001, Inglis and Tigano 2006). *Paecilomyces* is polyphyletic across three ascomycete orders, the Eurotiales, the Hypocreales and the Sordariales (Luangsa-ard et al. 2004, Inglis and Tigano 2006). The type species, *Paecilomyces variotii,* and thermophilic relatives from the section *Paecilomyces* belong to the order Eurotiales (*Trichocomaceae*), whereas mesophilic species of the section *Isarioidea* and related to *Paecilomyces farinosus* are in the order Hypocreales (*Clavicipitaceae* and *Hypocreaceae*). In the Eurotiales anamorph *Paecilomyces* species are related to the teleomorphs *Talaromyces* and *Thermoascus* (Inglis and Tigano 2006). Only one species, *Paecilomyces inflatus,* has affinity for the order Sordariales (Luangsa-ard et al. 2004). Within the order Sordariales, *P. inflatus* is found to be associated with two ascomycetes, namely *Chaetomium globosum* and *Neurospora crassa* (Luangsa-ard et al. 2004).

Paecilomyces spp. are found in a great range of habitats, substrates and materials, including soils, litter, compost, sewage sludge, lakes, mouldy grain, straw and wood (Samson 1974, Domsch 1980, Harney and Widden 1990, Polishbook et al. 1996, del Rio et al. 2001, Ryckeboer et al. 2003b). These fungi prefer aerated habitats for growth and reproduction, but are also capable of surviving in the anaerobic mullet gut (Walsh 1972, Mountfort and Rhodes 1991). As a saprophyte *Paecilomyces* spp. normally obtain nutrients from decaying organic matter, but they can also derive nutrients from living cells of insects as a parasite (Siddiqui and Mahmood 1996). *Paecilomyces* spp. can readily grow and reproduce over a wide temperature range from 5 to 55°C (Samson 1974, Pitt and Hocking 1999, Maheshwari et al. 2000, van Heerden et al. 2002).

The genus *Paecilomyces* has received only a little attention in lignocellulose degradation studies, despite the abundance of these fungi in agricultural wastes at different stages of decomposition (Tuomela et al. 2000). Although there are a few studies dealing with the degradation of lignocellulose components by these fungi, the results are partially contradictory and their enzymatic mechanisms are hardly understood (Kapoor et al. 1978, Kainsa et al. 1979, Mishra et al. 1979, Ghanen 1991, del Rio et al. 2001, Martinez et al. 2005*). P. variotii* efficiently degrades cellulose and lignin in wheat straw causing an increase in humus-like substances (Mishra et al. 1979). The ability to degrade cellulose and lignin in wood by *Paecilomyces* sp. has been discussed by Eslyn et al. (1975). These authors observed that the fungus depleted lignin more rapidly then other cell wall components.

Table 4. Degradative activities of microfungi isolated from compost for different lignocellulose components

Fungus	Subdivision	*Source material	Degraded compound				Reference
			Lignin	Cellulose	Hemicellulose	Wood	
Aspergillus fumigatus	Ascomycotina	WC, HC	+	++	+++	+	Flannigan and Sagoo 1977, de Vries and Visser 2001
Chaetomium thermophilum var. coprophile var. dissetum	Ascomycotina	MSW, MC, WC	lacc[a]	+++	+	+	Chefetz et al. 1998, Hakulinen et al. 2003, Li et al. 2003
Emericella nidulans (anamorph: Aspergillus nidulans)	Ascomycotina	MC,SC, GC	-	++	++ (arabinoxylan)	-	Fernandez-Espinar et al. 1994, Chikamatsu et al. 1999
Fusarium oxysporum	Ascomycotina	GC	+	++	++	+	Falcon et al. 1995, Rodriguez et al. 1996b, Abdel-Sater and El-Said 2001
Fusarium solani	Ascomycotina	MC, GC	++	++	++	+	Rodriguez et al. 1996b, Gopinath et al. 2005, Lozovaya et al. 2006
Humicola grisea var. thermoidea	Deuteromycotina	MSW, WC, HM	nd	+++	+++	+	De-Paula et al. 1999, Pocas-Fonesca et al. 2000, Salles et al. 2005

Malbranchea cinnamonea (= Malbrachnea pulchella = Theromoidium sulfereum)	Deuteromycotina	MC, HC	+	+++	++	nd	Jain et al.1979, Matsuo and Yasui 1985,
Melanocarpus albomyces (= Myriococcum albomyces)	Ascomycotina	MC	lacc[a]	++	+++	nd	Prahbu and Maheshwari 1999, Saraswat and Bisaria 2000, Kiiskinen et al. 2002 Miettinen-Oinonen et al. 2004
Myceliophthora thermophila (= Sporotrichum thermophile)	Ascomycotina	MC, GW	nd	+++	++	nd	Ofosu-Asiedu and Smith 1973b, Bhat and Maheswari 1987, El-Naghy et al. 1991, Topakas et al. 2003
Paecilomyces sp.	Deuteromycotina	MSW, MC, HM, GC	+	++	++ (xylan)	+	Eslyn et al. 1975, Okolo et al. 1998, del Rio et al. 2001, Martinez et al. 2005
Paecilomyces variotii	Deuteromycotina	MC	+	++	+++ (xylan)	+	Kelly et al. 1989, Dix and Webster 1995, Gopinath et al. 2005
Penicillium chrysogenum (= Penicillium notatum)	Ascomycotina	GC	+	+	+ (xylan)	nd	Rodriguez et al. 1994, Falcon et al. 1995, Rodriguez et al. 1996a
Preussia fleischhakii	Deuteromycotina	GC	+	nd	nd	+	Haider and Trojanowski 1975
Rhizomucor pussilus (= Mucor pusillus)	Zygomycotina	MC, HC	nd	+/-	+	nd	Dix and Webster 1995, Rahman et al. 2001

Scytalidium thermophilum (=Torula thermophila)	Deuteromycotina	MC	+	+++	nd	+	Jain et al. 1979, Ögel et al. 2001, Ögel et al. 2006
Talaromyces emersonii	Ascomycotina	MC, SC, WC, GC	+	+++	+	+	Ofosu-Asiedu and Smith 1973b, Tuohy et al 1993, Murrey et al. 2001
Talaromyces thermophilus	Ascomycotina	GC, MC, HC	+	+	+	+	Dix and Webster 1995
Thielavia terrestris (= Allescheria terrestris)	Ascomycotina	MC, GC	+	++	nd	+	Ofosu-Asiedu and Smith 1973b, Eslyn et al. 1975, Gilbert et al. 1993
Theromoascus aurantiacus	Ascomycotina	MSW	+	+++	+++	(+)	Machuca and Duran 1993, Kalogeris et al. 2003, Milagres et al. 2003
Thermomyces lanuginosus (= Humicola lanuginosa)	Deuteromycotina	MSW, SC, GC	+	+/-	+++ (xylan and arabinoxylan)	nd	Waksman et al. 1939, Jain et al. 1979, Puchart et al. 1999, Damaso et al. 2000
Trichoderma koningii	Deuteromycotina	MSW, GM	++	+++	++ (xylan and arabinoxylan)	+	Gerber et al. 1997, Lopez et al. 2006

* Source material of compost: MSW= municipal solid waste, MC = mushroom compost, SC = straw compost, HM = manure, horse compost, WC = wood compost, HC= hay compost, GC = garden compost, FC = food compost, GM = grape marc

[a] lignin degradation not determined, but found to produce laccase, phenol oxidase

nd = not determined

About 50 % of lignin loss caused by *P. variotii* in beech sawdust has been reported, and the rate of lignin degradation dependes on culture conditions and the composition of fermentation medium (Ghanem 1991). In contrast, recent studies investigating hardwood decay inoculated with *Paecilomyces* sp. using analytical pyrolysis - GC/MS revealed an increase in lignin proportion due to preferential removal of polysaccharides (del Rio et al. 2001, Martinez et al. 2005). In studies by Calvo et al. (1995), effluent from the paper industry treated with *P. variotii* had a very high alkali lignin loss (78 %). However, a significant proportion of effluent alkali lignin was found to be attached to the fungal biomass. Lignin-related compounds such ferulic, syringic and *p*-coumaric acids and other phenols, are rapidly degraded by *Paecilomyces* spp. (Rauhoti et al. 1989, Ghosh et al. 2006, Mukherjee et al. 2006, Sachan et al. 2006). Recently *Paecilomyces lilacinus* was found to transform and even completely mineralize biphenyl (Gesell et al. 2001) and dibenzofuran (Gesell et al. 2004). In addition *P. variotii* is able to utilize toluene as the sole carbon source and degrade it to CO_2 (Estevez et al. 2005).

Most of P*aecilomyces* spp. produce a range of glucanases that hydrolyze hemicelluloses and celluloses (Kelly et al. 1989, Almeida e Silva et al. 1995, Okolo et al. 1998, Tribak et al. 2002, Yang et al. 2006). Some of these enzymes have been purified and characterized (Kelly et al. 1989, Okolo et al. 1998). *Paecilomyces farinosus* (Fakoussa and Frost 1999), *Paecilomyces* sp. (Donnison et al. 2000) and *P. variotii* (Rahouti et al.1989) also secrete laccase-type phenol oxidases when they grow on phenolic compounds.

P. variotii has been utilized in the industrial process for the production of microbial protein. In this process known as "Pekilo", the fungus is grown on a variety of lignocellulosic wastes, such as wood hydrolyzates, spent sulphite liquor, molasses and vinasse (Romantschuk and Lehtomäki 1987). The resulting protein produced contains all the essential amino acids for animal feed.

1.2. Lignocellulosic materials and their degradation

In nature, lignocellulose containing biomass is the major source of renewable organic matter produced by plant photosynthesis. Lignocellulosic wastes are formed in plant production (agriculture and forestry) and industrial processes (pulp and paper). It accounts for about 60 % of the total plant biomass produced on earth (Perez et al. 2002).

Lignocellulose is physically hard, dense and recalcitrant to degradation. However, it is an extremely rich and abundant source of carbon and chemical energy, therefore the recycling of carbon involving lignocelluloses is essential to maintain the global carbon cycle (Malherbe and Cloete 2002). Chemically, lignocellulose is a combination of two linear polymers, cellulose and hemicellulose and a nonlinear, three-dimensional polymer lignin (Perez et al. 2002). Cellulose is surrounded by matrix like hemicellulose and encrusting lignin (Figure 3).

1.2.1. Lignin

Lignin is the most abundant high-molecular mass aromatic compounds in plants. High levels of lignin in plants are synthesized into wood and account for 15–36 % of the dry weight of wood whereas in grass it is less than 20 % . Lignin is complex of phenolic polymers that reinforce the walls of certain cells in the vascular tissues of higher plants

(Figure 4.) Lignin plays important roles in plants involving: mechanical support, water transport and in protecting cellulose and hemicelluloses from microbial attack by physical exclusion by reducing the surface area available of threse to enzymatic attack (Eriksson et al. 1990).

The lignin polymer arises from enzyme-initiated oxidation of three phenolic precursors coumaryl, coniferyl and sinapyl alcohols, which differ in their degree of methoxylation. These precursors are synthesized from L-phenylalanine and L-tyrosine, generated via the shikimic acid metabolic pathway, where the compounds are initially derived from CO_2 fixed by plant photosynthesis (Higuchi et al. 1977). Lignin precursors and their relative amounts vary significantly between the plant species. Softwoods contain mainly guaiacyl lignin, hardwood both guaiacyl and syringyl type lignin, whereas lignin in grasses consists of all three units (guaiacyl, syringyl and hydroxyphenol lignin) (Sjöström 1993).

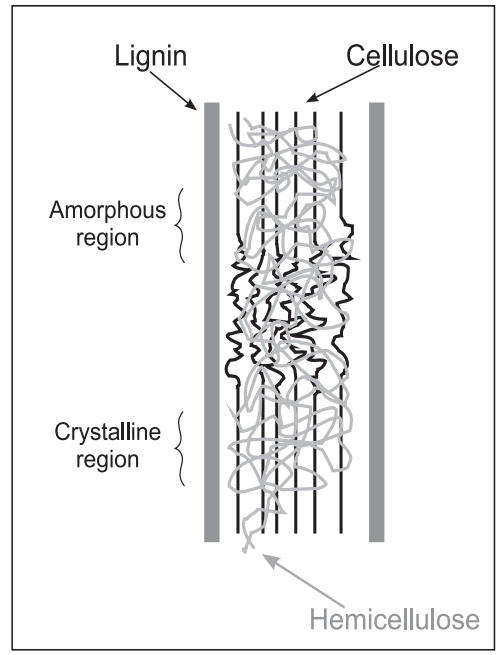

Figure 3. A schematic structure of lignocellulose

Lignin formation results in an almost random series of bonding, and therefore the lignin polymer have no single repeating bond between these subunits.The most frequent inter-unit linkage is the β-O-4 (β-aryl ether). It is also the one most easily cleaved chemically, providing a basis for industrial processes such as chemical pulping (Singh 2006). The other linkages are β-5, β-β and 5-5 are more resistant to chemical degradation (Sjöström 1993, Argyropoulos and Menachem 1997). At least 10 different types of aryl ether and carbon-carbon bonds are known to link phenylpropanoid units together (Argyropoulos and Menachem 1997). Recently a new type of linkage in softwood forming dibenzodioxocin moiety was discovered by Brunow and co-workers (Brunow et al. 2001).

Due to its complicated structure, lignin is highly resistant to microbial degradation and its association with cellulose and hemicellulose polysaccharides also imparts degradation resistance to these polymers (Hatakka 2001). Several properties of lignin account for its resistance to microbial attack: it is a water-insoluble, aromatic, three-dimensional molecule containing non-hydrolyzable bonds (Brunow 2001). Moreover, the enzymes needed for the complete degradation of lignin are only induced in the absence of readily available nutrients. Thus degradation of lignin is delayed and only occurs slowly.

The complex structure and the properties of the lignin polymers make studies on their degradation difficult. Isolation of native lignin is complicated if it is at all possible (Hatakka 2001) and therefore, suitable model compounds are needed for study. This problem can be overcome by using ^{14}C-labeled lignin preparations, i.e. the dehydrogenation polymer (DHP). The chemical properties of DHP resemble those of natural lignin.

^{14}C-labeled DHP can be prepared by polymerizing specifically or uniformly labeled coniferyl alcohol with horseradish peroxidase (Kirk and Brunow 1988), resulting in guaiacyl (G-type) lignin. The G-type lignin - synthetic or natural is more recalcitrant to breakdown than other types of lignin (Faix et al.1985). ^{14}C-labeled synthetic lignins make it possible to follow and measure the fate of the ^{14}C-label (mineralization, solubilisation) during microbial degradation.

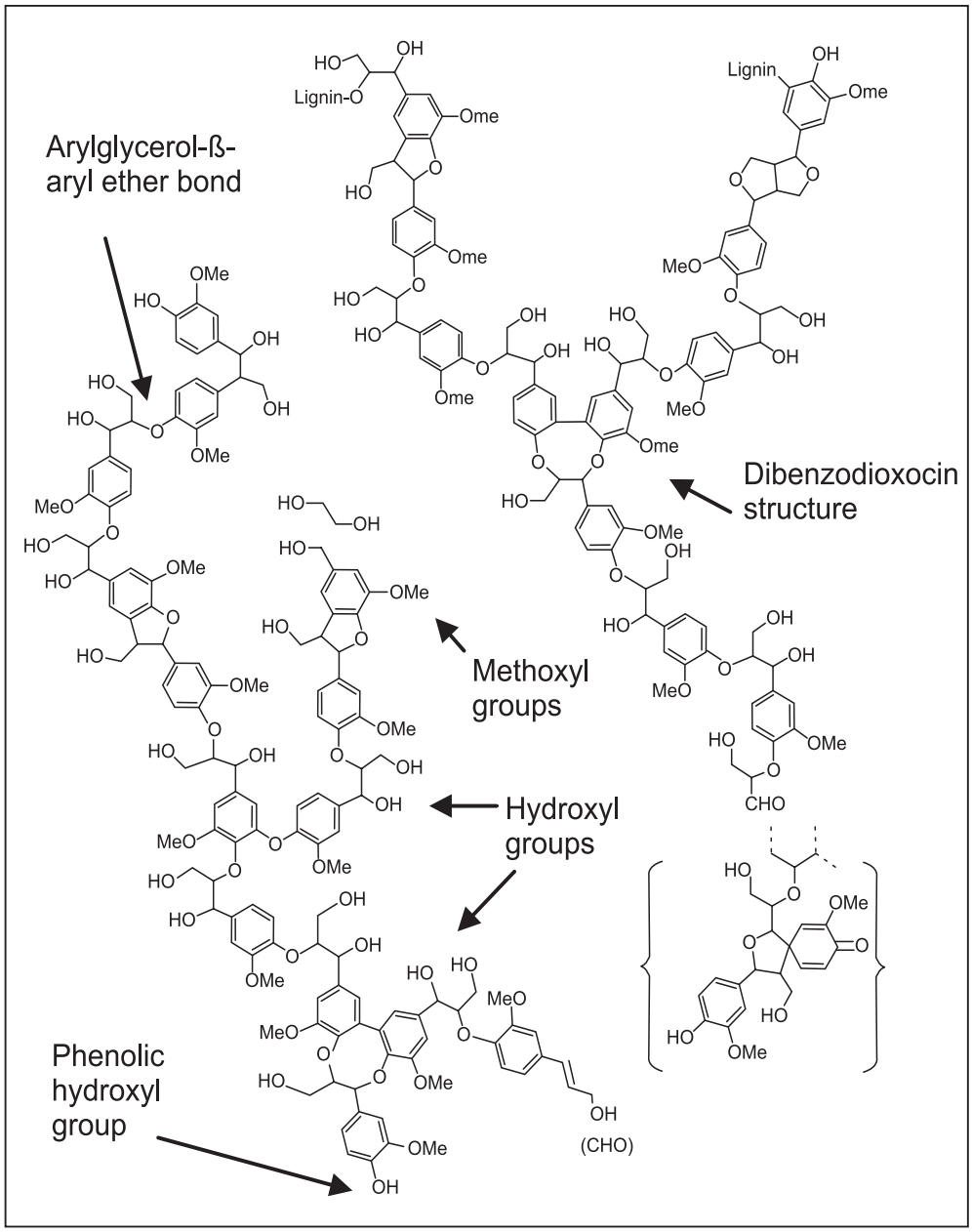

Figure 4. Schematic structure of spruce lignin, showing the common functional group (Brunow 1998)

Several chemical procedures have also been introduced for the estimation of lignin content (Tuomela et al. 2000). Some of these methods are more suitable for quantitative lignin analysis such as Klason lignin, Acid insoluble lignin, Kappa number, whereas others i.e., Kraft lignin more applicable for isolating lignin for biodegradation studies. The determination of Klason lignin is the most common method used to analyse lignin quantitively. In this method, hydrolysis of plant cell walls by sulphuric acid (70 %) dissolves all lignocellulose components other than lignin.The residual material is considered to be lignin (Dence 1992). However, the Klason method is subject to errors if it is used for determining lignin content in plants that contain other interfering high molecular weight substances such as proteins and tannins (Hammel 1997). The presence of humic substances (HS) formed during biological decomposition in compost or soil may also lead to error in the Klason lignin determination.

1.2.2. Lignin biodegradation

The biological degradation of lignin is an important contributor to the earth's carbon cycle, because most renewable carbon is either in lignin form or in compounds protected by lignin from enzymatic degradation (Hatakka 2001). Lignin degradation is also responsible for wood destruction and may have an important role in plant pathogenesis (Lozoyova et al. 2006). On the other hand, lignin degrading organisms and their enzymes are of special interest and might be used in many industrial processes such as in pulp and paper technology and also for the treatment of many organopollutants, stains and dyes.

The most efficient lignin degrading microorganisms are taxonomically related to basidiomycete white rot and litter decomposing fungi (Hatakka 2001). However, some ascomycetes, mitosporic, brown rotting and mycorrhizal fungi and some bacteria also contribute to lignin degradation (Daniel and Nilsson 1998, Hatakka 2001). Under aerobic conditions, lignin is decomposed considerably but in anaerobic environments lignin losses are negligible (Kirk and Farrell 1987).

Unlike microbial degradation, abiotic degradation or transformation may also occur in special environments and under special conditions, such as those that arise from alkaline chemical spills (Blanchette 1991) or UV radiation (Vähätalo et al. 1999). In a forest ecosystem: temperature, moisture content and pH are the major factors influencing lignin transformation and breakdown activities (Donnelly et al. 1990 Criquet et al. 2000). The abiotic oxidation by transition metals such as Cu, Ni and Zn in calcareous soils also participates in the incorporation of phenolic and lignin related compounds into humus (Kaschl et al. 2002) and Mn-oxalate complex in cooperation with xylanase can modify the structure of plants cell wall (Lequart et al. 2000).

1.2.2.1. Lignin -degrading microorganisms

Although the carbon content in lignin is high, microorganisms are unable to utilize polymeric lignin as a sole source for carbon and energy (Kirk et al. 1976). It is generally believed that lignin depolymerization is necessary to gain access to cellulose and hemicellulose. Presumably, this is the real purpose for lignin biodegradation. During sugar utilization from polysaccharides of wood, H_2O_2 is produced by the action of glucose oxidase and glyoxyl oxidase (Kirk and Farrell 1987, Hatakka 2001) and this is a prereq-

uisite for degradation by white rot fungi. White rot fungi are the most efficient lignin degraders known so far. They can completely break down the lignin of wood by the enzyme-mediated oxidation of lignin referred as "enzymatic combustion" (Kirk and Farrell 1987). Fungal attack is an oxidative and non-specific process, which decreases methoxyl, phenolic and aliphatic content of lignin, cleaves aromatic rings and forms new carbonyl groups (Kirk and Farrell 1987, Hatakka 2001). These changes in the lignin molecule result in depolymerization and carbon dioxide production (Kirk and Farrell 1987). The lignin degradation by white rot fungi is faster than that of other micro-organisms in nature. However it varies between species (Hatakka 2001).

White rot fungi secrete an array of extracellular enzymes i.e. lignin peroxidases (LiP), manganese peroxidases (MnP) and laccases. In many basidiomycetous white rot fungi, lignin degradation occurs during secondary metabolism, i.e. under conditions of nutrient limitation. The limiting nutrient for fungal growth in most wood and soils is probably nitrogen (Kirk and Farrell 1987). It was suggested that N-limited growth conditions are natural for fungi, since wood contains only low levels of nitrogen (Kirk and Farrell 1987). However, there are variations in nitrogen metabolism between fungal species. The addition of organic nitrogen to the growth medium represses lignin-degrading activity in *Phanerochaete chrysosporium* (Keyser et al.1978) yet it stimulates biomass yields and laccase production in *Bjerkandera* sp. and *Trametes pubescens* (Kaal et al. 1993, Galhaup et al. 2002). Thus, lignin degradation is greatly influenced by the presence of nitrogen. Increasing the oxygen tension in cultures has a strong multiple activating effects on lignin degradation (Kirk and Farrell 1987). High oxygen levels (100 %) enhance lignin mineralization in *Phlebia radiata* during growth on poplar wood (Hatakka and Uusi-Rauva 1983).

To date, little is known about the degradation of lignin by other microorganisms other than white rot fungi. Brown rot fungi which taxonomically belong to basidiomycetes minimally alter the lignin via hydroxylation and demethylation reactions that result in a loss of strength in the woody biomass along with a rapid loss of cellulose and hemicellulose (Blanchette 1995, Hatakka 2001). The presence of wood stimulated demethylation activity of lignin model compounds by brown rot *Gloeophyllum trabeum,* which was able to evolved 30–60 % of $^{14}CO_2$ from nonphenolic (4-O$^{14}CH_3$)-labeled β-O-4 dimer (Niemenmaa et al. 1992).

In a study using litter decomposing fungi from the genera *Agrocybe* and *Stropharia* the mineralization of ^{14}C-(ring)-labeled synthetic lignin (DHP) was about half of the level obtained with white rot fungi (Steffen et al. 2000). Species such as *Marismius quercophilus* and *Mycena inclinata* were able to bring about a 60 % decrease in lignin content in oak leaves (Steffen et al. 2007) The most studied litter decomposing edible fungus *Agaricus bisporus* degrades as much as 35 % of lignin over an 80 day of cultivation period (Durrant et al.1991). *Cyathus bulleri* from the family *Coprinaceae* has been reported to degrade lignin (Abbott and Wicklow 1984) and because it produces low levels of cellulases and xylanases (Saxena et al. 1994) it is considered as a selective lignin degrader.

Some ectomycorrhizal fungi (*Cenococcum, Amanita, Tricholoma and Rhizopogon*) mineralize ^{14}C-labeled synthetic lignin and corn stalk lignin slowly. However, the efficiency of this process falls far behind that of white rot fungi (Trojanowski et al. 1984, Haselwandter et al. 1990).

Bacterial lignin degradation has been most extensively studied in filamentous actinomycetes that belong to the genus *Streptomyces*. These gram-positive bacteria can solubilize less than 45 % of the total lignin present in water-soluble acid-precipitable polymeric lignin (APPL) and mineralize 3 % of the C-label over 21 days (Berrocal et al. 1997, Crawford et al. 1983). During growth in solid state culture of wheat straw *Streptomyces cyaneus* produces a laccase-type phenol oxidase and the activity of the enzyme was found to correlate with both solubilization and mineralization rates (Berrocal et al. 1997, Berrocal et al. 2000).

1.2.2.2. Lignin -degrading microfungi

The degradation of lignin by microfungi has only been studied by a few researchers. Some soil - and wood inhabiting fungi degrade lignin but the extent of degradation is limited compared to that of white rot fungi. The ascomycete *Daldinia concentrica* was able to bring about a 40 % decrease in lignin content (Nilsson et al. 1989) whereas *Chrysonilia sitophila* decreased the lignin content of pine wood by only about 25 % (Ferraz and Duran 1995). Lignin is mostly modified by demethylation i.e. removal of methoxyl groups (Eslyn et al. 1975). Analysis of decayed wood after the fungal growth indicates that oxidative C_{α} –C_{β} and β-O-aryl cleavages occurred during lignin degradation (Ferraz and Duran 1995).

Rodriguez et al. (1996b) observed a significant decrease in lignin in wheat straw by several ascomycetes and mitosporic fungi. Furthermore studies performed using isotopic methods confirmed the lignin degrading capacities of microfungi. Some degradation of differentially labeled DHPs by soft-rot microfungi of the strains *Preussia*, *Chaetomium* and *Stachybotrys* have been reported by Haider and Trojanowski (1975). The total release of $^{14}CO_2$ in 10 or 15 days was only about 2–4 % of the total added radioactivity. Nevertheless, *Chaetomium piluliferum* released 30 % $^{14}CO_2$ from differently labeled corn stalk lignin over seven weeks (Haider and Trojanowski 1980). In contrast to white rot fungi, microfungi preferably degrade DHP and corn stalk lignin in a high nitrogen medium (Haider and Trojanowski 1975, 1980). In similar degradation studies involving *Penicillium chrysogenum, Fusarium oxysporum, F. solani and Pestalotia oxyanthi*, the fungi were able to mineralize up to 2.5–9 % and 4.5–7 % of ^{14}C-labeled lignin within 28 days, depending on the label in the synthetic lignin uniformly or side chain label (Rodriguez et al. 1994, Falcon et al. 1995). In contrast to white rot fungi, the degradation was maximal during primary metabolism. Some wood-rotting ascomycete species are also capable of mineralizing lignin and lignin model compounds to some extent (Liers et al. 2006). This activity has been reported for *Xylaria* species that are capable of causing white rot-like decay accompanied by substantial lignin loss (Pointing et al. 2003).

Several authors suggest that biodegradation of lignin by microfungi might be, at least partly, brought about by extracellular enzymes. In fact, different types of lignin-degrading enzymes have been detected in several studies with ascomycetes. Thus, laccase was reported in *Coniochaeta* (Barbosa et al. 1996), *Hortaea acidophila* (Tetsch et al. 2005), *Fusarium proliferatum* (Regalado et al. 1999), *Mauginiella* sp. (Palonen et al. 2003), *Penicillium chrysogenum* (Rodriguez et al. 1996a) and *Xylaria* (Liers et al. 2006). Peroxidases have been reported for the ascomycete *Chrysonilia sitophila* (Rodriguez et al.1997), *Aspergillus terreus* LD- 1(Kanayama et al. 2002), *Coniochaeta ligniaria* NRRL 30616 (Lopez et al. 2007). How-

ever, these enzymes may not be so efficient in oxidizing lignin as those of white rot fungi, though they may have special properties. Hence, a thermophilic strain *Thermoascus aurantiacus,* which degrades 15 % of lignin of *Eucalyptus gradis* and bleaches *Eucalyptus* kraft lignin (Machuca and Duran 1993, Machuca et al. 1998) produces high levels of phenol oxidase (Machuca et al. 1998). Phenol oxidase of *T. aurantiacus* has a capability to oxidize efficiently a range of substrates typical of phenoloxidases in the absence of H_2O_2 over an acidic pH range (2.6–3.0) and at elevated temperatures up to the 70–80°C range (Machuca et al. 1998). *Fusarium proliferatum,* which is able to mineralize synthetic lignin, also secretes superoxide radicals during lignin mineralization (Regalado et al. 1999). Superoxide radicals may generate highly reactive hydroxyl radicals, which are known to be involved in lignin degradation (Guillen et al. 2000). Only low levels of laccase activity were required for lignin degradation by *Petriellidium fusoideum*, where the activity was also correlated with the production of hydroxyl radicals (Gonzalez et al. 2002).

Fungi are also important in the degradation of lignin in aquatic habitats. Sutherland et al. (1982) reported 4–5 % mineralization of ^{14}C-labeled maple and spruce lignin over 30 days by several species of marine fungi. The facultative marine ascomycete *Sordaria fimicola* mineralized 10 % of synthetic lignin and was able to produce lignin-modifying enzymes when grown in the low nitrogen medium supplemented with sea salts (Raghukumar et al. 1996).

1.2.3. Lignin degrading enzymes

1.2.3.1. Characteristic of lignin-degrading enzymes

Lignin biodegradation is a process involving the action of oxidative enzymes and in subsequent chemical reactions (Hatakka 1994, 2001). Reactions catalyzed by enzymes play a significant role in the complete degradation of lignocellulose biomass. Since the lignin polymer is large and highly branched, lignin-degrading mechanisms must be extracellular and unspecific. The presence of stable ether and carbon-carbon bonds in lignin requires oxidative rather than hydrolytic enzymes. Due to the irregular structure of lignin the degradative enzymes must have lower substrate specificity than typical biological catalysts (Hammel 1997).

Some of the enzymes secreted by fungi generate hydrogen peroxide as an oxidant and others transfer the electrons. The most important lignin-modifying biocatalysts are lignin peroxidases (LiPs), manganese peroxidases (MnPs), functional hybrids of both enzymes (versatile peroxidases VP) and laccases (phenol oxidases). All extracellular peroxidases and laccases have the ability to catalyze one-electron oxidation resulting in the formation of radicals, which undergo several spontaneous reactions. These, in turn lead to various bond cleavages including aromatic ring fission (Kirk and Farrell 1987, Hatakka 2001). Apparently, these enzymes act using low-molecular mass mediators to carry out lignin degradation.

1.3.2.2. Peroxidases

LiP and MnP are heme-containing proteins, which require hydrogen peroxide as an oxidant. The lignin-degrading system depends on low molecular mass metabolites and cofactors. A secondary metabolite, veratryl alcohol (3, 4-dimethoxylobenzene) is a redox

mediator for LiP, whereas Mn^{+2}, which is ubiquitous in all lignocelluloses and in soil is a redox mediator for MnP. Some of most important features distinguishing these enzymes from other oxidoreductases are their very low pH optima and much higher redox potentials (Hatakka 2001, Hofrichter 2002).

LiP was first found in the lignin-degrading fungus *Phanerochaete chrysosporium* during secondary metabolism under nutrient-limited culture conditions. LiP is produced by several white rot fungi such *Phlebia radiata* (Hilden et al. 2006), *Trametes trogii* (Vares and Hatakka 1997) and *Bjerkandera* sp. BOS55 (ten Have et al. 1998). To date, there is limited knowledge on LiP production in fungi other than white rot basidiomycetes. However, three isoforms of LiP has been purified from the ascomycete *Chrysonilia sitophila* (Rodriguez et al. 1997). LiP-like peroxidase from the lignite degrading fungus *Penicillium decumbens* P6 has recently been characterized (Yang et al. 2006).

The substrates of LiP include both phenolic and non-phenolic aromatic compounds. The phenolic substrates are oxidized to yield products similar to those produced by peroxidases, while oxidation of nonphenolic methoxybenzenes is unique to LiP (Kersten et al. 1985). The oxidation of these substrates to yield aryl cation radicals can result in either demethylation, C_α - C_β cleavage of lignin model compounds, benzylic alcohol oxidation, or hydroxylation of aromatic rings and side chains (Kirk and Farrell 1987).

MnP is secreted by a distinct group of wood white rot and soil litter basidiomycetes. However, recently Kanayama et al. (2002) purified an alkaline MnP-like peroxidase from the ascomycete *Aspergillus terreus* LD-1. This enzyme seems to be very attractive to the pulping industry since it has unique pH optima of between pH 11 and 12.5.

MnP oxidizes Mn^{2+} to Mn^{3+} using H_2O_2 as the oxidant. The product of Mn^{+2} oxidation, Mn^{+3} must be chelated by organic acids such as oxalate or malonate , which are produced by the fungus (Galkin et al. 1998, Hofrichter et al. 1999). With the help of these chelators Mn^{+3} ions are stabilized promoting their release from the enzyme into materials such wood. Chelated Mn^{+3} acts as a strong oxidant that preferably attacks phenolic moieties of lignin resulting in the formation of free radicals that tend to disintegrate spontaneously (reviewed by Hofrichter 2002). However, in the presence of different unsaturated fatty acids and their derivates, nonphenolic lignins are oxidized through a MnP - lipid system (Kapich et al. (1999a, 1999b). Unlike lignin, purified MnP also oxidizes HS from litter and brown coal (Steffen et al. 2002, Hofrichter and Fritsche 1997) and HS synthesized from catechol (Hofrichter et al.1998, Steffen et al. 2002) in addition to several organopollutants (Steffen et al. 2003).

The third type of peroxidase called versatile peroxidase (VP) has also been reported to be secreted from *Pleurotus eryngii* (Camarero et al. 1999) This peroxidase is capable of the oxidative reaction, characteristic of both LiP and MnP.

1.3.2.3. Laccase

Laccase is a phenol oxidase, which belongs to the blue multicopper oxidases. These enzymes catalyze one-electron oxidation of four reducing-substrate molecules concomitantly with four-electron reduction of molecular oxygen to water. Laccases typically contain four copper atoms of three types that can be identified on the basis of their spectroscopic and paramagnetic properties. The presence of different copper domains are important for the catalytic activity of laccases. The type-1 Cu bound via two His and

one Cys as ligands, functions as the primary electron acceptor. This extracts electrons from the reducing phenolic substrates and transfers them to the trinuclear centre at the Type-2 and Type-3 Cu sites. The trinuclear centre is typically coordinated by eight His residues and is the binding site for the second substrate, i.e. molecular oxygen. This oxygen atom accepts electrons from the Type-1 Cu site for its subsequent reduction to water (Claus 2003, Baldrian 2006; Figure 5).

A typical laccase has a molecular mass of about 60–80 kDa. However, enzymes from ascomycetes *Monocillium indicum* and *Gaeumannomyces graminis* appear to be substantially larger with molecular mass of 100–190 kDa (Thakker et al. 1992, Edens et al. 1999). Laccase mostly exhibits isoelectric points (p*I*) and pH optima in the acidic pH range (Bollag and Leonowicz 1994). However, laccases of some soil-inhabiting basidiomycetes (Schneider et al. 1999) and ascomycetes (Chefetz et al. 1998a, Robles et al. 2000) including *Rhizoctonia praticola* (Bollag and Leonowicz 1994), *Coprinus cinereus* (Schneider et al. 1999) and *Chaetomium thermophilum* (Chefetz et al. 1998a) have higher pH optima over a 7–8 pH range. Temperature profiles of laccase shows optima ranging between 30–60°C (Gianfreda et al. 1999).

Laccases are mostly inducible enzymes and their induction has been observed at the level of transcription and translation upon addition of copper, and also aromatic compounds such xylidine (Collins and Dobson 1997, Palmieri et al. 2000, Litvintseva et al. 2002, Tetsch et al. 2005). However, the repression of laccase at the high concentrations of these compounds due to their toxic effect has also been demonstrated in some fungi (Bollag and Leonowicz 1984, Eggert et al. 1996).

Natural and synthetic lignin in addition to industrial lignins such as lignosulfonates or indulin AT are good elicitors of laccase production. Lignocellulosic residues in growth media significantly increase laccase formation in several fungi (Ardon et al.1996, Machuca et al. 1998, Pickard et al. 1999, Lorenzo et al. 2002).

Laccase has a rather low specificity as regards to reducing substrates. Therefore a number of quite different organic and inorganic compounds including diphenols, polyphenols, substituted phenols, diamines and aromatic amines are readily oxidized (Thurston 1994). Laccase catalyzes the cleavage of the C_α- C_β bonds in phenolic $_\beta$-1 and $_\beta$-O-4 lignin model dimers by oxidizing the C_α and by splitting the aryl-alkyl bonds (Eriksson et al. 1990).

Laccase in not a key enzyme in lignin degradation, as lignin contains only 10–15 % of phenolic structures in wood (Singh 2006). However, in the presence of aromatic electron-transfer mediators such as ABTS, the blue laccase from *Coriolus* (*Trametes*) *versicolor* becomes capable of oxidizing non-phenolic substrates (Bourbonnais and Paice 1990). The same effect was exerted by the laccase secreted by *Pycnoporus cinnabarinus* in the presence of the fungal metabolite 3-hydroxyanthranilate (Eggert et al. 1996).

Laccases are produced by higher plants and fungi, but they are also found in bacteria, yeasts and insects (Thurston et al. 1994, Claus 2003). In plants, laccases are involved in the formation of lignin, whereas in bacteria laccases are involved in melanin production and spore coat resistance (Castro-Sawinski et al. 2002, Martins et al. 2003).

Laccase-like activities have also been detected in the cuticle of larval and adult stage of insects, during the sclerotization process (Hopkins and Kramer 1992).

The majority of laccases characterized so far have been derived from fungi especially from white rot basidiomycetes, ascomycetes and mitosporic fungi. Well-known laccase producers are phytopathogenic ascomycetes such as *Botrytis cinerea* (Slomczynski et al. 1995), *Gaeumannomyces graminis* (Edens et al. 1999), *Magnaporthe grisea* (Iyer and Chattoo 2003), *Ophiostoma novo-ulmi* (Binz and Canevascini 1997) and *Mauginella* sp. (Palonen et al. 2003). Laccase production was also reported for some: soil ascomycete species from the genera *Aspergillus*, *Fusarium* and *Penicillium* (Rodriguez et al. 1996b, Scherer and Fischer 1998, Regalado et al. 1999), compost *Chaetomium thermophilum* (Chefetz et al. 1998a), freshwater ascomycetes (Abdel-Raheem 1997, Junghanns et al. 2005) and from lichenized ascomycetes of the order *Peltigerales* (Laufer et al. 2006, Zavarzina and Zavarzin 2006). The crystal structure of laccase found in ascomycete *Melanocarpus albomyces* has already been solved (Hakulinen et al. 2002).

The wood-degrading ascomycete *Bothryosphaeria* that is closely related to the wood-rotting basidiomycetes constitutively produces a dimethoxyphenol-oxidizing laccase (Vasconcelos et al. 2000), which is significantly induced by veratryl alcohol (Barbosa et al. 1996, Dekker et al. 2001). Several ascomycete species involved in the decay of plant biomass in terrestrial habitat and salt marshes have been shown to have laccase genes and to oxidize syringaldazine (Lyons et al. 2003, Pointing et al. 2005). *Trichoderma* strains have also been reported to produce syringaldazine-oxidizing polyphenoloxidases (Assavanig et al. 1992). These are mainly associated with spores, which may act in the morphogenesis of this fungus (Assavanig et al., 1992; Hölker et al., 2002). Two *Xylaria* strains i.e., *X. polymorpha* and *X. hypoxylon* exhibit ABTS oxidizing laccase in complex liquid media and solid birch wood cultures (Liers et al. 2006).

Fungal laccases also contribute to pigment production, fruiting body formation and plant pathogenesis (Thurston 1994, Gianfreda et al.1999). They are found to be involve in various ecological processes in soil, forest litter and compost environments and they have often been isolated from these habitats (Criquet et al. 2000, di Nardo et al. 2004, Chefetz et al. 1998b). In the soil and compost environments, released laccases are capable of both polymerization and depolymerization of humic acids, and may contribute to carbon cycling (Stevenson 1994, Chefetz et al. 1998b, Zavarzina et al. 2004). Soil orga-

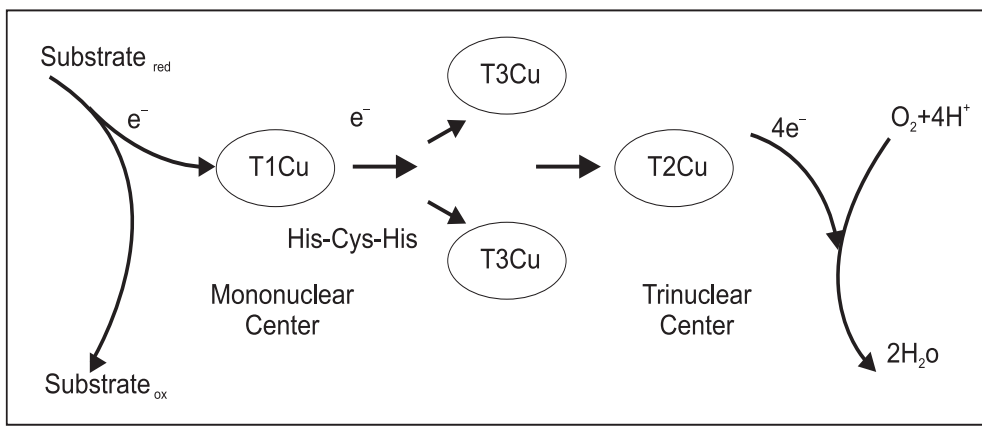

Figure 5. Catalytic cycle of laccase modified from Baldrian (2006)

nopollutants may also be oxidized by laccase to less toxic polymers, which after various transformation may be incorporated in the soil humus (Gianfreda and Bollag 1994).

1.2.4. Cellulose and hemicellulose

Cellulose and hemicelluloses act as both structural and energy-storage components of plants. Cellulose is the most abundant, insoluble and highly ordered renewable polymer found in nature (Lynd et al. 2002). It is the major constituent of plant cell walls providing their rigidity (Beguin and Aubert 1994). Cellulose comprises approximately 30–40 % of dry wood weight and 45 % of the dry weight of grasses. The polymer consists of D-glucose subunits linked by β-1, 4 bonds, forming long insoluble chains (microfibrils) linked together by hydrogen bonds and van der Waals forces. The microfibrils are grouped together to make up the cellulose fiber. The degree of polymerization of cellulose chains ranges from 500 to 25 000 (Kuhad et al. 1997). Cellulose is often crystalline in the native stage and is surrounded by a mixture of amorphous cellulose (non-organized chains), hemicellulose and lignin. Because of its structural rigidity, crystalline cellulose is resistant to the action of individual cellulases. Effective conversion of cellulose to monosaccharides is therefore only possible by the synergistic action of these enzymes (Figure 6). Amorphous regions occur near the crystal surface and are prone to enzymatic attack (Beguin and Aubert 1994). The crystallinity index, and the nature of substances (lignin) with which the cellulose is associated are the most important factors affecting the speed of cellulose degradation (Kuhad et al. 1997).

Hemicelluloses, the third most abundant constituents of plant cell walls found in nature, represent about 20 to 35% of the lignocellulose dry mass. They are heterogeneous polymers of pentoses (D-xylose, L-arabinose), hexoses (D-mannose, D-glucose, and D-galactose) and sugar acids. They are linked together by β-1,4- glycosidic bonds, but β-1,3-, β-1,6-, α -1,2-, α- 1,3- and α -1,6- glycosidic bonds are also reported (Sjöström 1993). Hemicelluloses are chemically associated with or cross-linked to other polysaccharides, proteins and lignin.Moreover, they form a matrix together with pectin and proteins in primary cell walls and with lignin in secondary cell walls. The matrix of lignin and hemicellulose encrusts and protects the cellulose of the plant cell wall (Hammel 1997).

The major hemicelluloses of hardwood and annual plants are xylans (15–30%), which probably interact with lignin and cellulose more than any other hemicellulose (Kuhad et al. 1997). The main hemicelluloses of softwood are galactoglucomannans (15–20%), arabinoglucoroxylan, and arabinogalactan (Sjöström 1993). Since hemicelluloses have an amorphous nature and a lower degree of polymerization (approximately 70–200) they are degraded more easily than cellulose (Kuhad et al. 1997, Perez et al. 2002).

1.2.5. Cellulose and hemicellulose biodegradation

The ability to produce cellulolytic enzymes is widespread among microorganisms, but only a limited number of species are actually able to degrade native cellulose in its crystalline form. Cellulolytic microorganisms (fungi and bacteria) can establish synergistic relationships with non-cellulolytic species in cellulosic wastes (Maheshwari et al. 2000). The interaction between both populations leads to a complete degradation of cellulose,

which releases CO_2 and water under aerobic conditions, or carbon dioxide, methane and water under anaerobic conditions (Béguin and Aubert 1994).

The cellulolytic enzyme system of fungi consists of a number of extracellular endoglucanases, cellobiohydrolases and *β*-glucosidases, which work together catalyzing the hydrolysis of cellulose (Lynd et al. 2002). Endoglucanases (EGs) represent less than 20 % of the total protein in *Hypocrea jecorina* (anomorph *Trichoderma reesei*) and preferentially hydrolyse cellulose microfibrils in the amorphic parts of the fibril, releasing new terminal ends. Cellobiohydrolases (CBHs), which may account for 20 to 60 % of the total cellulase proteins of fungi act on the existing or endoglucanase-generated chain ends. In *H. jecorina* CBH I attacks reducing ends and CBH II the non-reducing ends of the fiber (Teeri 1997). CBHs and EGs can degrade amorphous cellulose, but CBHs are the only enzymes that efficiently degrade the crystalline form of cellulose. Both enzymes release cellobiose molecules (reviewed by Teeri 1997). The effective hydrolysis of cellulose also requires *β*-glucosidases (BGL), which break down cellobiose and release two glucose molecules. Products of cellulose degradation are made available as carbon and nitrogen sources for either cellulolytic organisms, or other microbes living in the environment containing cellulosic materials.

Fungal cellulases are the most widely investigated so far. Cellulases from microfungi have been studied more than those of any other physiological group, and their cellulases currently dominate the industrial applications of cellulases (Nieves et al. 1998). In particular, the cellulase system of *Hypocrea jecorina*, (formerly known as *Trichoderma reesei)*) has been the focus of research for 50 years. The full genera sequence of this fungus is published. H. *jecorina* produces at least two cellobiohydrolases (CBHI and CBHII), five endoglucanases (EGI, EGII, EGIII, EGIV, and EGV), and two *β*-glucosidases (BGLI and BGLII; Kubicek and Penttilä 1998, Nevalainen and Penttilä 2004). The cellulase system of the thermophilic fungus *Humicola insolens* is homologous to the *H. jecorina* system and also contains several enzymes (Schülein 1997).

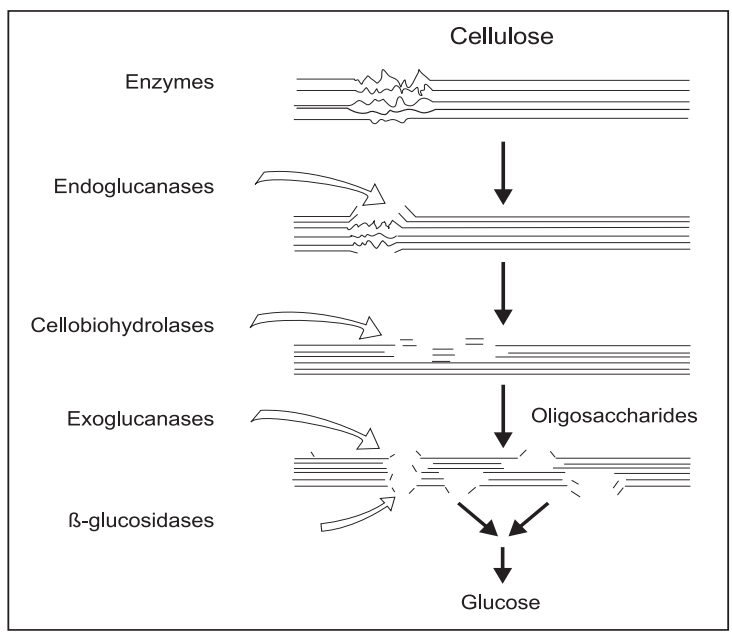

Figure 6. A simplified schematic of complete enzymatic hydrolysis of a cellulose microfibril by fungi modified according to Lynd et al. (2002)

A 28-kDa endoglucanase (EG28) from white rot fungus *Phanerochaete chrysosporium* has different properties from the common type of cellulase (Henriksson et al. 1999). EG28 lacks the ability to bind crystalline cellulose and is active with xylan and mannans, suggesting a possible role in lignocellulose degradation. The *P. chrysosporium* genome reveals an impressive array of genes encoding cellulases including 40 endoglucanases, seven cellobiohydrolases and at least nine *β*-glucosidases (Martinez et al. 2004).

Cellulases are inducible enzymes, and their biosynthesis is enhanced markedly in media containing inducers (Béguin and Aubert 1994, Aro et al. 2005). Low constitutive amounts of cellulases can be found in saprophytic fungi, which makes it possible for them to attack cellulose when available and thus lead to the formation of the inducer activity of cellulase biosynthesis. Therefore the transcription of *H. jecorina* EG and CBH genes present in the low levels prevailing under in the non-induced conditions can be induced up to at least 1100-fold in the presence of cellulose (Carle-Urioste et al. 1997). Cellobiose and sophorose (β-1, 2-glucobiose) are also good inducers, whereas glucose is a strong repressor of endoglucanases activity (Béguin and Aubert 1994, Ilmén et al. 1997, Kubicek and Penttilä 1998). When rice and wheat straw were used as carbon sources *Neurospora crassa, Melanocarpus* sp. MTCC 3922, *Scytalidium thermophilum* MTCC 4520 and *Myceliophthora* sp. IMI 387099 were able to initiate cellulase production(Romero et al. 1999, Kaur et al. 2006, Badhan et al. 2007). In contrast, when ammonium salts were used as nitrogen source cellulase production in *Aspergillus fumigatus* and *Thermoascus aurantiacus* were initiated (Steward and Perry 1981, Kalogeris et al. 2003).

In fungi the production of cellulases may also be regulated by factors other than induction and repression by sugars. Several lignin-related aromatic compounds, which are found in association with cellulose in nature, stimulate or inhibit cellulase production. This feature is common to some wood-rotting white and brown rot basidiomycetes such as *Phlebia radiata, Trametes gibbosa, Trametes* (*Coriolus*) *versicolor, Postia placena and Gloeophyllum trabeum* (Müller et al. 1988, Highley and Micales 1989, Tsujiyama et al. 2003).

The hydrolysis of hemicellulose requires similar types of enzymes to those required for cellulose hydrolysis. However, more enzymes are required for the complete degradation of hemicellulose, because of the greater complexity of hemicellulose compared to cellulose (Malherbe and Cloete 2002). Hemicellulases hydrolyze glycosidic linkages in hemicelluloses and are classified according to their substrate specificities. The total enzymatic degradation of hemicellulose polymers requires the action of "endo-type" enzymes that liberate short oligosaccharides, which are subsequently degraded by side-group cleaving enzymes and "exo-type" enzymes and finally monomeric sugars and acetic acid are formed. Similar to the cellulases, hemicellulases act synergistically. Of all these enzymes xylanase is the best studied (reviewed by Kuhad et al. 1997).

Xylanases have been found in many ecological niches, where plant material is present. Complete breakdown of a branched xylan requires the action of several enzymes, particularly endo-1,4-*β*-xylanases and *β*-xylosidases. Endoxylanases are able to cleave the xylan backbone into smaller oligosaccharides including xylobiose, which can then be degraded further by *β*-xylosidases into D-xylose (Biely and Tenkanen 1998). Both classes of enzymes ain addition to their encoding genes have been found in many microorganisms. Xylanases of *Trichoderma* spp. and *Aspergillus* spp. are the most studied (reviewed by Biely and Tenkanen 1998 and by de Vries and Visser 2001). However, xylanases have also

been described in several basidiomycetous white rot (Rogalski et al. 1993, Maijala 2000, Khalil 2002), brown rot (Ritschkoff et al. 1994) and in litter decomposing fungi (Steffen et al. 2007). As in the mesophilic fungi, a multiplicity of xylanases have been observed in some thermophilic fungi (Prabu and Maheswari 1999, Puhart et al. 1999, Latif et al. 2006, Yang et al. 2006, Badhan et al. 2007). Xylanases are the major group of industrial enzymes, that finds significant application in the paper and pulp industry. This is because the hydrolysis of xylan facilitates the release of lignin from paper pulp and hence reduces the usage of chlorine as the bleaching agents (Buchert et al. 1998).

1.3. Humic substances

1.3.1. Occurrence and formation of humic substance

The term humic substance(s) (HS)refers to high-molecular mass, dark brown material that is rich in aromatic compounds. It originates from plant, animal or microbial organic matter. They are the most widely distributed biosynthesizes products on the earth. Besides soils, humic substances (HS) can be found in lakes, rivers, compost, sediments, peat and lower-rank coals (Aiken et al. 1985).

HS comprise three fractions that are humic acids (HAs; Figure 7), fulvic acids (FAs; Figure. 8) and humin. The fractions differ in acid and base solubility. The physico-chemical characteristics of HS are shown in Table 5. HS mainly consist ofmolecules with aromatic rings connected by long aliphatic chains (Shevchenko and Bailey 1996). Moreover, HS fractions are presumed to have similar molecular structures, only showing differences in the degree of cross-linking between their macromolecules, the content of oxygen and the amount of hydroxyl, carbonyl or carboxyl groups (Kästner and Hofrichter 2001).

Lignin and polysaccharides are two major organic precursors of HS formation (Inbar et al. 1989). The glycosidic link between lignin and polysaccharides is broken first. Changes in lignin begin with the loss of phenolic and methoxyl groups with an increase in carboxyl and carbonyl groups (Inbar et al. 1992, Shevchenko and Bailey 1996). Thus HS are less aromatic and contain fewer methoxyl but more carboxyl groups than lignin (Shevchenko and Bailey 1996). The process in which organic matter is transformed into humic substances (HS) is called humification. In composts the process forms humus-like materials which are permanent. Humification starts as early as the first mesophilic phase of composting, develops in the thermophilic phase and finally results in the production of humic substances in the maturing phase of the composting process. However, mature compost is only an intermediate product of humification because HS still undergo changes. Decomposition of high and low-molecular-weight plant components and synthesis of microbial cell constituents are involved in humification. During composting HS are formed in a relatively short period of time.

The humification of lignocellulosic waste depends on lignin oxidation, which mainly occurs during the thermophilic phase of composting (Baca et al. 1992). Phenoloxidases (peroxidases and laccase) catalyze this process by mediating oxidative coupling of phenolic products that result from biomass decomposition (Chefetz et al 1998a, Dec and Bollag 2000, Zavarzina et al. 2004). During plant degradation 70 % of organic carbon is mineralized. At the same time lignin is covalently bound to HS (Shevchenko and Bai-

ley 1996, Almendros et al. 2000). Humification reactions still continue, after molecules chemically bound together. Thus, lignin is in fact being degraded at the same time as some of it is being polymerized (Shevchenko and Bailey 1996). In the beginning of the composting process HAs are formed from aliphatic compounds, which are later replaced by aromatic molecules with a high proportion of oxygen and nitrogen constituents (Miikki et al. 1997, Veeken et al. 2000, Sanchez-Monedero et al. 2002). Compost HAs are less aromatic, contain fewer carboxyl and oxygen-containing functional groups and they have lower molecular mass than soil HAs (Inbar et al. 1990, 1992, Garcia-Gil et al. 2004, Liguirati et al. 2005). Elemental composition, functional group contents and E_4 / E_6 ratio (indicate changes in molecular mass) analyses of HAs from various composts and soil are shown in Table 6. The composition of compost HAs resembles those of plant residues, peat and incompletely humified materials (Inbar et al. 1990, 1992).

Humic acids represent the most abundant fraction of humic substances. HAs are a complex of polymers in which phenolic and aliphatic units are linked by peptides, amino acids, amino sugars and other organic constituents. HAs can be recovered from soil, litter, compost or low-rank coal such as lignite or brown coal; as sodium salts (Na humates) by sodium alkaline (Hofrichter and Fakoussa 2001a). These are subsequently precipitated with hydrochloric acid at pH 2 (Senesi and Loffredo 2001). Due to HAs high molecular mass and heterogeneous structure comprising aromatic building blocks, they are resistant to microbial attack (Haider and Martin 1988, Willmann and Fakoussa 1997b).

1.3.2. Biodegradation of humic substances

Resistance to biodegradation is one of the most important properties of humic substances. Since soil humus generally decomposes at the rate of 2–5 % a year (Linharse and Martin 1978), the age of the HS seems to play an important role for the metabolism and mineralization of these compounds. Because of their size, humic compounds cannot be taken up into the hyphae, thus an extracellular enzymatic activity is assumed (Kästner and Hofrichter 2001) These enzymes may either polymerize HS under certain conditions in addition to degrading them (Chefetz et al. 1998a, Zavarzina et al. 2005).

A variety of techniques can be used to follow the bioconversion of HS such as microbiological, spectrophotmetric, gravimetric and radioactive methods. Nowadays, one of the best and easiest methods available to study the degradation of HS is the application of synthetic radioactively labeled HS preparations. Synthetic HS are prepared from ^{14}C-labeled polyphenols such as ^{14}C-labeled ^{14}C-catechol, ^{14}C-glucose or ^{14}C-glycine by the oxidative polymerization or the Millard reaction (Blondeau 1989, Hofrichter et al. 1998b). However, it should be kept in mind that the synthetic model of HS may differ from the naturally occuring HS. Moreover, the mineralization of synthetic compounds does not reflect the whole degradation process, which occurs in natural environments. Extracted natural HS can also be used in degradation studies and these are often analysed by HPSEC (high pressure size exclusion chromatography). HPSEC is used to evaluate changes in the relative molecular weight distribution of soluble HS and gives indications of the absolute molecular masses of humic and fulvic acids (Blondeau 1989).

Table 5. Chemical and physical characteristics of humic substances (Stevenson 1994, Shevchenko and Bailey 1996, Kästner 2000)

Humic substance (HS)	Color	Solubility	Molecular mass (MM) kDa
Humic acid (HA)	dark brown	soluble in alkali	1.4–100
Fulvic acid (FA)	yellowish	soluble in alkali and in acid	0.5–30
Humin	black	insoluble in alkali and in acid in all pH	similar to MM of HA

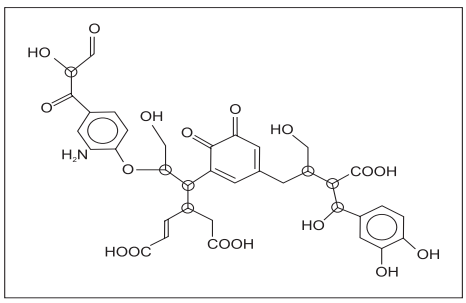

Figure 7. (above) Model structure of humic acid Stevenson (1994)

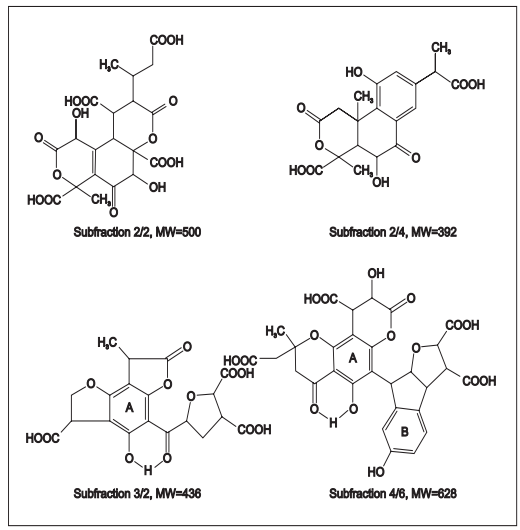

Figure 8. (right) Structural models of selected subfractions of fulvic acids according to Leenheer & Rostad (2004; MM = molecular weight in Daltons).

Numerous organisms are able to slowly decompose humic acids slowly. The most active degraders are basidiomycetous fungi and actinobacteria, which are also efficient lignin degraders.

Degradation of HA is similar to that of lignin, that occurs under co-metabolic conditions, i.e. in the presence of easily assimilable and metabolizable carbon sources such as glucose and pentoses (Blondeau 1989). However, exceptions exist (Řezàčovà et al. 2006).

The release of $^{14}CO_2$ from ^{14}C-HA and decolourization i.e. conversion of high molecular-mass (MM) HA into low molecular-mass FA of natural HA under co-metabolic conditions was observed in liquid glucose-mineral salt cultures of the basidiomycetes *Phanerochaete chrysosporium* and *Trametes versicolor* (Haider and Martin 1988, Blondeau 1989, Dehorter and Blondeau 1992), *Collybia dryophila* (Steffen et al. 2002) and the actinobacterium *Streptomyces viridisporus* (Kontchou and Blondau 1992). These were found to degrade HAs and form lower molecular mass FAs. The first successful study on the degradation of HAs was performed by Hurst et al. (1962), who reported that wood-colonizing basidiomycetes such as *T. versicolor* and *Hypholoma fasciculare* were capable of decolourizing soil HA. Soil HA is also decolourized in the presence of an additional carbon source such as glucose by isolates of *Streptomyces* (Kontchou and Blondeau 1992, Dari et al. 1995). In bacterial cultures the decolourization was partially caused by adsorption of HA onto the

bacterial cells surface, and partially caused by extracellular non-selective enzymes (Adhi et al. 1989).

The efficiency of fungi in modifying humic substances is considered to be associated with their extracellular non-specific enzyme system, especially lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Kästner and Hofrichter 2001). Addition of HA to fungal growth media may either stimulate enzyme activities (Dehorter and Blondeau 1992, Willmann and Fakoussa 1997b, Steffen et al. 2002) and RNA expression (Scheel et al. 2000), or inhibit them (Ralph and Catcheside 1994). Thus, in soil the absorption of laccase on humic constituents may change enzyme availability and activity (Criquet et al. 2000, Claus and Filip 1990, Kang et al. 2002). However, Keum and Li (2004) suggest that the inactivation of laccase by HA is not due to binding to humic material but to the dissociation and chelation by HA of copper from the active centre of the enzyme.

Table 6. Elemental composition, functional group contents and E4 / E6 analysis of humic acids (HAs) from different composts and soil (Aiken et al. 1985, Chen et al. 1996, Unsal and Ok 2001)

Constituent	Humic acids origin				
	Composts				
Elemental composition	Municipal solid waste (MSW)	Sewage sludge (SS)	Wood compost (WC)	Grape waste (GW)	Average of different soils
C (%)	52.0	53.0	50.6	58.2	56.2
H (%)	6.0	5	5.6	5.9	4.7
O (%)	37.2	36.7	nd	29.0	32.2
N (%)	6.3	4.2	3.5	5.8	1.9
H / C [a]	1.4	1.2	1.3	1.2	1
C / N	11.7	13	16.9	14.2	20
Total acidity[b]	13.5	21.9	16.3	13.1	6.7
COOH[d] (meq g^{-1})	1.9	1.2	2.2	1.7	3.6
Phenolic-OH[d] (meq g^{-1})	11.6	20.7	14.1	11.4	39.0
E_4 / E_6 [*]	7.1	2.2	4.5	3.3	2.1

[a] represents HA aromaticity (a low ratio indicates an aromatic structure)
[b] sum of carboxyl and phenolic groups
[*] E_4 / E_6 (the ratio between absorbance at 465 nm and 665 nm, the higher ratio indicates a lower molecular mass of HA)
[d] functional group
nd = no data

High activities of ABTS and syringaldazine oxidizing enzymes (MnP, laccase) detected during the process of HA depolymerization by *Nematoloma frowardii* b19 in the agar medium, indicates the direct involvement of lignin-modifying enzymes in the degradation process (Hofrichter and Fritsche 1997). However, it is interesting to note that depolymerization occurred over the course of several days. Other authors have demonstrated the important role of white-rot laccases in the disintegration of HA. (Frost and Fakoussa 1999, Zavarzina et al. 2004).

Among all lignin-modifying biocatalysts involved in HA degradation, MnP is considered to be a key enzyme. The ability of MnP derived from white rot fungus *Nematoloma frowardii* to mineralize humic macromolecules *in vitro* has been demonstrated in study using ^{14}C-HA prepared from ^{14}U-catechol (Hofrichter et al. 1998b). Approximately 17 % of ^{14}C-labeled HA is released as $^{14}CO_2$ within one week of enzymatic treatment. Other *in vitro* studies (Wunderwald et al. 2000, Steffen et al. 2002) confirm that MnP is able to depolymerize and mineralize HAs. A positive correlation between decolourization of HA and MnP production has been also reported in many wood and straw degrading basidiomycetes (Gramss et al. 1999). However, the brown rot fungus *Fomitopsis pinicola,* which does not produce extracellular lignin-degrading enzymes can alter molecular masses of HA as effectively as the wood-decaying white rot fungus *Hypholoma* (*Nematoloma*) *frowardii* by producing lignin-modifying enzymes (Gramss et al 1999).

The role of white rot fungi in the degradation of humus in nature is unclear. White rot fungi grow preferentially in compact wood but they compete poorly with soil and compost-dwelling microorganisms. Moreover, soil HS were even found to inhibit the growth of white rot fungi (Rayner and Boddy 1988). The pH in soils and compost is usually higher and comparable to that in wood whereas the C/N ratio is lower than reported for optimal degradation of lignin and HAs by wood-colonizing white rot fungi. However, two white rot fungi isolated from biosolids compost namely *Trametes* sp. M23 and *Phanerochaete* sp.Y6, have the ability to bleach compost HA during growth under solid- state conditions using perlite as a solid support (Granit et al. 2007). The strong bleaching ability of coal derived HA (lignite) by these fungi has been reported previously by Frost and Fakoussa (1999).

Litter decomposing fungi, which are taxonomically and physiologically related to white rotting fungi that occupy soil and humus layers of forests have also been shown to disintegrate high-molecular mass HS of forest litter efficiently (Steffen et al. 2002). *Collybia dryophila* can degrade both soil HA and synthetic ^{14}C-HA prepared from catechol and it secretes MnP that is involved in the mineralization process. In contrast, only traces of a synthetic HA-like polymer were mineralized by the ectomycorrhizal symbionts of Douglas fir (*Pseudotsuga menziesii* [Mirb.] Franco; Durral et al.1994). Nevertheless, humic substances stimulate root colonization and the production of mycelium in the mycorrhizal fungus *Glomus claroideum* BEG 23 (Gryndler et al. 2005).

The utilization of HA has so far been studied in wood and litter-decomposing basidiomycetes (Blondeau 1989, Dehorter and Blondeau 1992, Steffen et al. 2002) whereas the involvement of microfungi in this process has been studied only rarely. According to Kästner and Hofrichter (2001) none of the six *Penicillium* sp. strains isolated from different soils was capable of utilizing HA as the sole carbon source. In another study *Aspergillus awamori, Penicillium* sp. and *Humicola* (*Thermomyces*) *insolense* could decolourize forest soil

HA under co-metabolic conditions and HA was also utilized as the sole carbon and / or nitrogen source by the fungi (Mishra and Srivastava 1986). Řezàčovà et al.(2006) did not find a significant effect of additional carbon source on the utilization of soil HA by microfungi. Gramss et al. (1999) detected the decrease of decolourization rates by about 25–50 % in media containing HA as the sole carbon source compared to supplemented media with malt extract. Higher fungal biomass and decolourization of litter HA by *Chalara longipes* has been observed in media supplemented with nitrogen (Koukol et al. 2004). Aquatic HA was degraded under co-metabolic conditions by *Cladosporium cladosporioides* isolated from bog lake water (Claus and Filip 1998). The degradation process was accompanied by an increase in the oxygen content of HS as well as by a relative decrease in aromatic carbon and an increase in aliphatic carbon (Claus and Filip 1998).

Laccase from *C. cladosporioides* does not decolourize HA to a significant extent only (5 to 10 %) even in the presence of enzyme mediator HBT (Claus and Filip 1998). The ascomycetes *Xylaria* sp i70, *Botrytis cinerea* H1 and mitosporic fungus *Alternaria* sp.G5 also produced extracellular enzymes, although they are not able to effect coal HA decolourization (Hofrichter and Fritsche 1996). However, *Paecilomyces farinosus* produced laccase and decolourized coal HA as efficiently as did some white rot fungi such as *Pleurotus ostreatus* (Frost and Fakoussa 1999). The ability to mediate the extensive solubilization of select low rank coals has also been reported for non- lignin degrading fungi such as *Aspergillus* spp., *Paecilomyces* sp., *Penicillium waksmani* and *Candida* spp. (Ward 1985, Scott et al. 1986, Steward et al. 1990). Recently, Yang et al. (2004) purified LiP-like peroxidase from the lignite-degrading microfungus *Penicillium decumbens,* which most probably is involved in the biodegradation process. The decolourizing/depolymerizing effect of purified LiP from *Phanerochaete chrysosporium* on coal macromolecules has been demonstrated earlier by Ralph and Catcheside (1999). Extracellular peroxidase, esterase and phenoloxidase enzymes also appear to be involved in coal solubilization by *Trichoderma* sp. and *Penicillium* sp. (Laborda et al. 1999). Interestingly, none of these enzymes were detected when fungi were grown in the absence of coal, which may indicate that they are coal-induced enzymes.

2. AIMS OF THE STUDY

The main aims of the work were:
(i) to examine of the ability of the compost-dwelling fungus *Paecilomyces inflatus* to modify and degrade the lignocellulose complex by each single components in addition to the humic substances under different growth conditions,
(ii) to identify and partially characterize the main extracellular lignocellulose degrading enzymes secreted by the fungus during biodegradation process,
(iii) to examine the degradation strategies of different *P. inflatus* strains under various conditions.

3. MATERIALS AND METHODS

3.1. Compost samples

Paecilomyces inflatus (Burnside) Carmichael strain BKT 02 was isolated from compost samples obtained from Ämmässuo municipal composting plant, Espoo, Finland. The compost was produced in an outdoor pile from vegetables, municipal waste, wood chips and newsprint by a conventional thermophilic process that lasted about six months. During this time the piles were turned periodically by machine. The maximum temperature during the composting was 65°C. The pH of the final product was 6.6 with a C/N ratio of 27. Three types of compost samples were used in this study. Some chemical and physical characteristics of the composts are given in Table 7.

Table 7. Some chemical and physical characteristics of the composts used in this study

Compost samples used	Characteristic				
	Age (months)	pH	C/N	Dry weight (%)	Klason lignin (% dw)
Compost (I)*	6.0	6.6	27	12.0	52
Compost (II, III)	3.0	6.3	24	10.8	56
Compost (IV)	2.0	7.1	15	9.1	40

* Refers to paper

3.2. Fungal strains

The anamorph of an ascomycete fungus *Paecilomyces inflatus* (Burnside) J.W. Carmich. strains BKT 01 and BKT 02 (DSM 16393, also called Comp-*Pi*) and 9931–03 were originally isolated at the Department of Applied Chemistry and Microbiology, University of Helsinki. *P. inflatus* 288.90 (Wood-*Pi*) and *P. inflatus* 684.96 (Rhizo -*Pi*) were obtained from the culture collection of Centraalbureau voor Schimmmelcultures collection, Baarn, The Netherlands. The fungal culture stocks were maintained on 2 % malt agar slants. The origin and characteristics of *P. inflatus* strains used in the study are presented in publication IV, Table 1.

3.3. Main experimental methods

The experimental set up and methods used are described in detail in the published papers I to IV and summarized in Table 8.

Table 8. Methods used in this work

Methods	Described and used in
Isolation of fungi using selective agar media	I
Agar plate screening tests with ABTS	I
Liquid cultivation of fungi	II, III, IV (and additional data)
Solid state cultivation of fungi	I, III, IV
Analytical methods	IV
Neutral detergent fibre, Acid detergent fibre	IV
Klason lignin	IV
Experiments with ^{14}C-labeled compounds	I, II
Extraction of ^{14}C-labeled compounds	I, II
Combustion of ^{14}C-labeled material	I, II
Spectrophotometric enzyme activity assays	I – IV (and additional data)
High performance size exclusion chromatography (HPSEC)	II (and additional data)

Methods not described in the publications I to IV but contributing to this research are presented in detail below.

3.4. Additional methods

3.4.1. Determination of molecular mass distribution of compost lignocellulose (unpublished)

Solid state cultures consisted of autoclaved (at 121°C for 20 min) compost (15 g; water content 50 %) in 100 ml Erlenmeyer flasks with cotton plugs and inoculated with 4 agar plugs (10 mm in diameter) of well grown fungal mycelia. The fungi were cultivated at 28°C for 46 days and then extracted with 40 ml of distilled water by sonication (3 min) and shaking for 30 min on a rotary shaker (120 rpm). After centrifugation samples were used for high-performance size exclusion chromatography (HPSEC) measurements.

HPSEC was used for the determination of the molecular mass distributions of lignocellulose fragments formed (Hofrichter et al. 2001). The high-performance liquid chromatography (HPLC) system (HP 1090 Liquid Chromatograph; Hewlett-Packard, Waldbronn, Germany), equipped with a diode array detector, was fitted with a HEMA-Bio linear column (8×300 mm, 10 μm) obtained from Polymer Standard Service (Mainz, Germany). The mobile phase consisted of 20 % acetonitrile and 80 % of an aqueous solution of 0.5 % (v/v) $NaNO_3$ and 0.2 % (v/v) K_2HPO_4. The pH was adjusted to 10 by the addition of 1M NaOH. The following separation parameters were used: flow rate 1 ml min^{-1}, detection wavelength 280 nm and injection volume 25 μl. Sodium polystyrene sulphonates (1.3 to 168 kDa; Polymer Standard Service) and biphenyl dicarboxylic acid (0.246 kDa) served as molecular mass standards.

3.4.2. Conditions for laccase production in liquid cultures (unpublished)

Czapek-Dox media described in paper I with different pH values were prepared with 0.1 M potassium phosphate buffer to follow the pH effect on the growth and enzyme production at the respective pH. Incubation was carried out in flasks containing 50 ml of medium at 28°C for 14 days. The effect of temperature was determined at temperatures ranging from 20 to 40°C using Czapek-Dox medium, pH 7.0.

Sodium nitrate, ammonium sulphate, ammonium phosphate, yeast extract and peptone were the nitrogen sources tested in laccase production experiments. They were added to Czapek-Dox medium (pH 7.0) and used at concentrations of 1 mg l^{-1} (Low N) and 5 mg l^{-1} (High N). Fungal cultures were incubated at 28°C for 25 days.

The effect of copper on the laccase production was determined using Czapek-Dox medium (pH 7.0) with high concentration of organic nitrogen (5 mg l^{-1}). The medium was supplemented either with 75 and 150 μM $CuSO_4$ after 4 days of fungal growth. The experiment was continued at 28°C for 30 days.

4. RESULTS

4.1. Lignin degradation (I and IV)

In order to evaluate the potential ligninolytic capability of the microfungi isolated from compost, an agar-plate screening was performed. The agar medium contained ABTS (2,2`-azinobis (3-ethylbenzthiazoline-6-sulphonate) as an indicator substrate for radical generating peroxidases and phenol oxidases. The most active fungi, i.e. the three strains of *Paecilomyces inflatus* that showed the characteristic green colouring of the agar medium (see Figure 9) were selected for lignin degradation and mineralization experiments with ^{14}C-labeled lignin (I).

Three *Paecilomyces inflatus* strains were found to degrade the synthetic lignin to some extent in an artificial compost environment under the following conditions: C/N = 15, pH 6.3, 28°C (I). The highest mineralization rates were obtained with *P. inflatus* BKT 02 (10 ± 1.1), *P. inflatus* isolate 2 (6.6 ± 0.5) and *P. inflatus* isolate 1 (6.2 ± 0.6) (I and unpublished). The cumulative $^{14}CO_2$ evolution by *P. inflatus* was most pronounced within the first three weeks of incubation and thereafter was almost linear until the end of experiment (Figure 10).

The mass balance of ^{14}C-DHP at the end of the experiment revealed that 15% of the initial radioactivity was recovered as water-soluble material (oxidized or modified lignin fragments) from fungal cultures. The rest of ^{14}C-DHP was incorporated into the residual compost and possibly into the fungal biomass and most probably contained the insoluble fractions of high-molecular mass

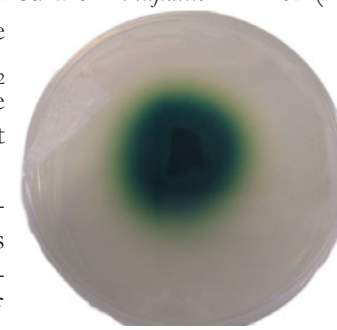

Figure 9. Photo of positive ABTS agar plate result with *Paecilomyces inflatus*

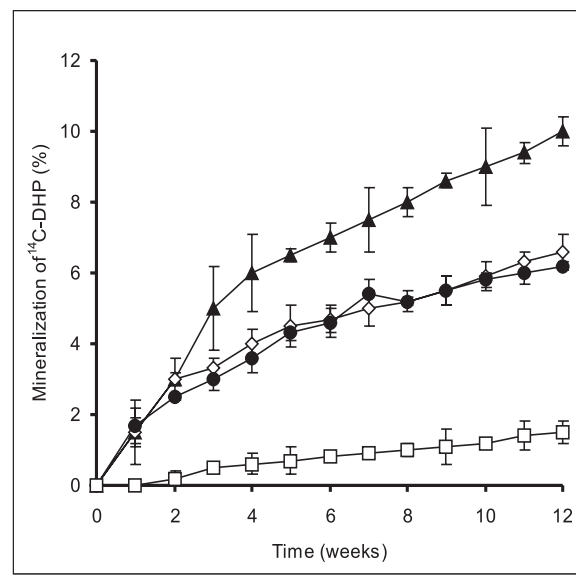

Figure 10. Release of $^{14}CO_2$ from $14C_\beta$-labeled lignin (80 000 dpm/flask) during the growth of three strains of *Paecilomyces inflatus* in compost. Strain BKT 01 *(open squares)*, isolate 3-9931 (black circles) and BKT 02 *(black triangles)* and uninoculated control *(black diamonds)*. Data were partially published in paper I and supplemented here by unpublished data. Data points represent means of three replicates (n = 3) with standard deviations *(bars)*.

lignins, which became like humin (I).

Degradation of unlabeled lignins in compost, straw and wood was evaluated by the determination of Klason lignin content using three *P. inflatus* strains originating from different environments. Details of *P. inflatus* strains have been published in paper IV. All *P. inflatus* strains were able to degrade lignin polymer from compost, straw and wood at different rates over the 12 weeks of experiment (IV, Tables 3–6). The results showed that as much as 15 % of lignin from compost was lost after 12 weeks of incubation. The lignin loss in compost was correlated with mycelial growth of compost-inhabiting *P. inflatus* and increased progressively until the end of the experiment (IV, Table 6). The lignin losses from straw, pine and birch wood were lower than from compost.

The compost`s lignocellulose content was investigated after 8 weeks of incubation with *P. inflatus* and by being subjected to gel permeation chromatography (unpublished)**.** High-performance size exclusion chromatography (HPSEC) was used for the determination of the molecular mass distribution of compost lignocellulose fragments formed after fungal treatment. *P. inflatus* released a small amount of water-soluble lignocellulose fragments of high molecular mass (~200 kDa) whereas the amount of medium size (25 to 30 kDa) and small fragments (0.6 kDa) in compost was slightly increased (Figure 11).

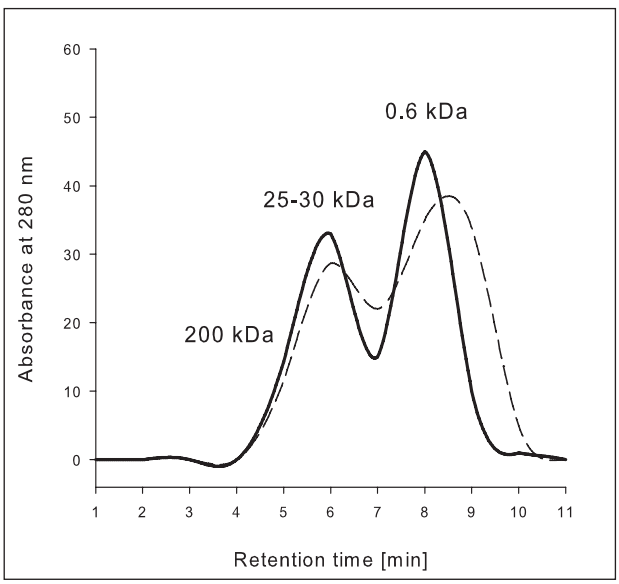

Figure 11. High-performance size exclusion chromatography (HPSEC): elution profile of soluble, partly aromatic lignocellulose fragments (absorption at 280 nm) extracted with water from compost inoculated with *P. inflatus (bold black line)* after 8 weeks of cultivation. Dashed line represents to an un-inoculated control.

4.3. Lignin-degrading laccase

P. inflatus produced laccases as the only ligninolytic enzymes in solid state and liquid cultures. MnP and LiP activities were completely lacking in all the investigated culture extracts.

Extracellular laccase could be measured under all tested growth conditions i.e. compost, wheat straw and spruce wood over the 12 week period of growth. Birch wood was the exception as with this substrate laccase was detected after only 8 weeks of incubation (IV, Figures 1a–d). The highest activity of laccase was recorded in Comp-*Pi* in compost with a maximum production of more than 44 ± 3.8 U g^{-1} in week 8 (IV, Figure 1d). In wood the enzyme production by compost strain started later and was lower (IV, Figures

1b–c). Rhizo-*Pi* produced considerable laccase activity at the beginning of incubation in straw samples with peak activity significantly higher than in the other two fungi (IV, Figure 1a).

In contrast to Rhizo-*Pi*, the production of laccase by Comp-*Pi* and Wood-*Pi* growing on lignocellulosic materials seemed to be growth associated and dependent on the culture conditions.

The oxidative activities of *P. inflatus* were studied in more detail in two liquid media, peptone and Czapek-Dox (I & unpublished). A number of different carbon sources were used in the laccase experiments in order to test their ability to promote growth and stimulate laccase secretion.

As presented in Table 9, xylan was an effective elicitor of laccase (37.0 ± 3.3 U l^{-1}) and supported fungal biomass growth in peptone containing liquid media. When *P. inflatus* was grown on a media containing cellobiose as the carbon source, it produced laccase activity of the same order of magnitude as when the fungus was cultured on xylose (18.5 ± 3.5 and 16.3 ± 1.9 Ul^{-1}; respectively). Media containing CM-cellulose and starch had no laccase activity, although they contributed to growth more than either cellobiose or xylose.

Insoluble carbon sources in peptone media also promoted laccase activity. The media were supplemented either with compost, wheat straw or wood (spruce and birch). High laccase activities were obtained during growth on compost and wheat straw. Laccase activities in the presence of spruce and birch wood were more than half of that observed for the compost medium. The onset of laccase activity occurred 7 days after inoculation in compost and straw. In contrast, in birch and spruce wood onsets were on days 14 and 28, respectively (Figure 12).

The effects of pH and temperature on laccase activity of *P. inflatus* are shown in Figures 13 and 14. The optimal initial pH and incubation temperature for laccase production by *P. inflatus* were determined to be pH 7.0 and 28 to 30°C, which are almost identical conditions as those found for the fungal growth (pH 6.5–7.5; 28°C).

The influence of different nitrogen sources was studied in order to increase laccase production by *P. inflatus*. For this purpose ammonium nitrate, ammonium sulphate, ammonium phosphate meat peptone and yeast extract were tested. Organic and inorganic nitrogen sources were added either at 1 g l^{-1} or 5 g l^{-1}. The studies were performed in a Czapek-Dox medium where glucose at concentration of 10 g l^{-1} was used as the carbon sources (unpublished). The highest enzyme production (39.0 ± 2.2 U l^{-1}) and most substantial

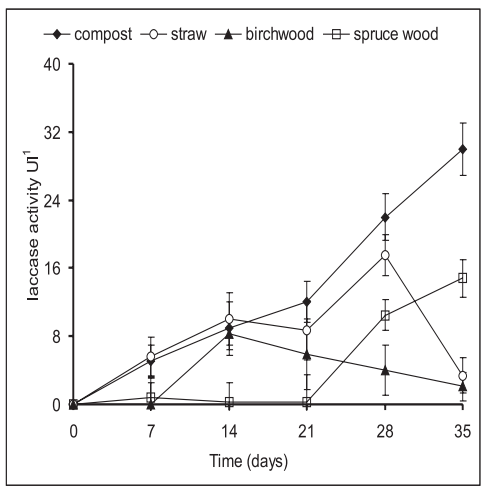

Figure 12. Time courses of enzyme activity of *P. inflatus* grown in peptone medium (pH 7.5) amended with compost extract *(black diamonds)*, milled wheat straw *(white circles)*, milled birch wood *(black triangles)* and milled spruce wood *(white squares)*. Each culture was conducted replicated three times and incubated at 28°C for 35 days.

Table 9. The effect of carbon and nitrogen sources on laccase production.

	Mycelium dry weight (mg 100 ml^{-1})	pH	Laccase activity (Ul^{-1})
Carbon source (10g l^{-1})			
None (control)	9.4 ± 1.1	7.1 ± 0.2	nd
Glucose	17.4 ± 2.3	7.2 ± 0.4	11.7 ± 0.8
Cellobiose	18.7 ± 0.5	7.1 ± 0.1	18.5 ± 2.7
Xylose	17.2 ± 3.6	7.2 ± 0.2	16.3 ± 4.1
Starch	25.9 ± 2.4	7.7 ± 0.3	nd
Citrus pectin	27.8 ± 4.1	4.0 ± 0.1	3.7 ± 3.2
CM-cellulose	28.3 ± 3.9	7.2 ± 0.4	nd
Birchwood xylan	nd	8.2 ± 0.1	37.0 ± 3.3
Nitrogen source (5g l^{-1})			
$NaNO_3$	18.7 ± 1.9	7.0 ± 0.4	12.3 ± 2.9
$(NH_4)_2SO_4$	14.8 ± 3.4	4.7 ± 0.3	5.7 ± 2.1
$(NH_4)_3PO_4$	15.4 ± 4.4	5.0 ± 0.2	6.9 ± 3.0
Meat peptone	32.0 ± 2.5	7.2 ± 0.4	40.0 ± 1.1
Yeast extract	22.6 ± 1.3	7.2 ± 0.1	29.1 ± 0.7

increase in fungal biomass were obtained when using meat peptone (5 g l^{-1}) N source. However, in nitrogen-limiting medium (1.0 g l^{-1} peptone) only low enzyme activity was detected (Figure 15). Replacement of peptone with yeast extract or ammonium salts resulted in subtantially decreased laccase formation as well as decreased growth of the fungus (Table 9). The pH in cultures supplemented with ammonia salts was 4.5–5.0 whereas in culture supplemented with peptone and nitrate the pH was about 7.1.

The effects of aromatic compounds veratryl alcohol, veratric acid, vanillin, vanillic acid and guaiacol (I) in addition to ionic copper (unpublished) on laccase production were studied. All aromatic compounds were added at a final concentration of 0.2 mM while copper at 75 and 150 μM to an actively growing culture of *P. inflatus* 4 days after inoculation. Among tested aromatics, vanillin and vanillic acids were found to be the best inducers for laccase production by compost strain *P. inflatus* isolate 2. Both represented a nearly 4-fold increase over the uninduced control (I, Figure 4).

The low concentration of copper sulphate (75 μM $CuSO_4$) stimulated laccase production. In cultures containing 75 μM $CuSO_4$, laccase activity appeared 14 days after inoculation and reached a maximum level (17.3 ± 2.4 U l^{-1}) on day 18 (Figure 16 A**)**. Furthermore, biomass production by *P. inflatus* was twice as high in a medium containing 75 μM $CuSO_4$ whereas concentrations over 150 μM seem to be toxic to the fungus, as significantly lower biomasses were determined (Figure 16 B) In cultures with high amounts of $CuSO_4$ (150 μM), laccase activity was detectable after 16 days with maximum activity of only 6.3 ± 3.2 U l^{-1}.

4.4. Cellulose and hemicellulose degradation

In general, all strains degraded all lignocellulose components in straw, birch, spruce and

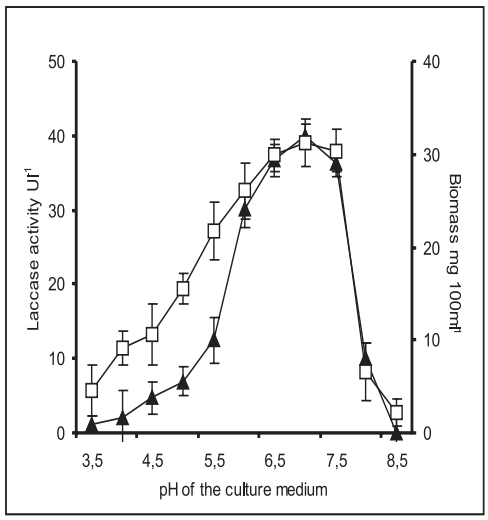

Figure 13. Effect of pH on laccase activity and biomass production by *Paecilomyces inflatus* (14 days old liquid cultures). *Black triangles* — laccase activity and *white squares* — biomass. Data points represent means of three replicates (n = 3) with standard deviations *(bars)*.

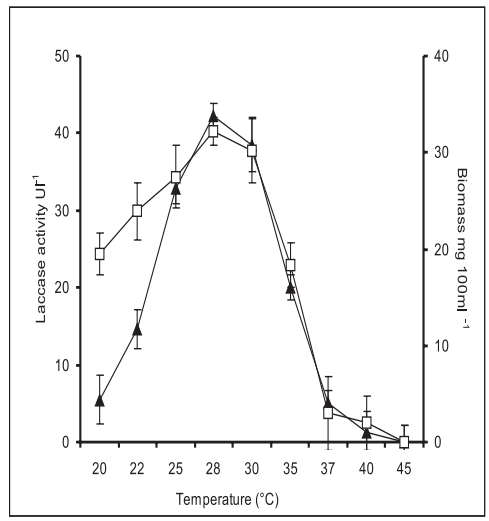

Figure 14. Effect of temperature on laccase activity and biomass production by *Paecilomyces inflatus* (14 days old liquid cultures). *Black triangles* — laccase activity and *white squares* — biomass. Data points represent means of three replicates (n = 3) with standard deviations *(bars)*.

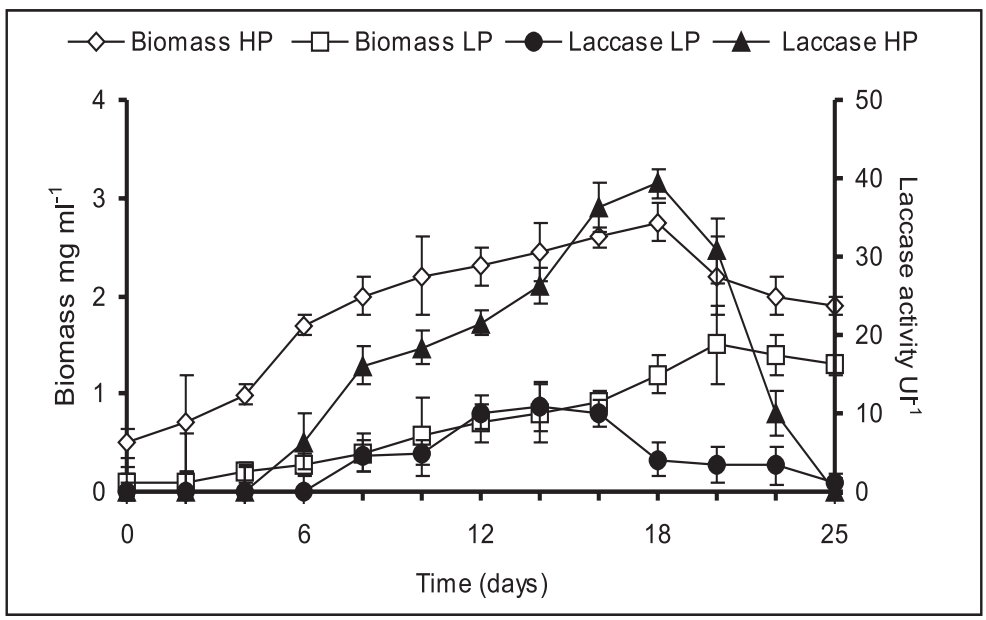

Figure 15. Time courses of laccase production by *P. inflatus* under low (LP; 1g l-1 of peptone) and high (HP; 5 g l-1 of peptone) nitrogen conditions using a Czapek-Dox basal medium. *Black triangles* refer to laccase activity under HP conditions, *black circles* - laccase activity under LP conditions, *white diamonds* - biomass in HP and *white squares* -biomass in LP cultures. Data points represent means of three replicates (n = 3) with standard deviations *(bars)*.

in compost. The strains had a nonselective degradation pattern, i.e. lignin was degraded at the same rate as cellulose and hemicellulose. Lignocellulose attack by all fungal strains led to substantial decreases in the cellulose components. Losses of cellulose from the lignocellulosic substrates were higher than that for lignin or hemicellulose and varied considerably in *P. inflatus* (IV, Tables 3–6). Comp-*Pi* and Rhizo-*Pi* caused the highest cellulose losses in both types of wood (40–60 %) whereas Wood-*Pi* did not reduce wood cellulose at all.

Wheat straw promoted the growth of all tested *P. inflatus* strains well. Strain Rhizo-*Pi* was the most efficient organism, and caused the highest mass losses (11 %) as well as the highest decay of lignocellulose components. Comp-*Pi* decomposed wheat straw at almost the same rates as Rhizo-*Pi* (IV, Table 3).

All studied *P. inflatus* strains caused higher total mass losses in spruce than in birch wood. However, differences were found in their ability to degrade the hemicellulose fraction in these wood types (IV, Tables 4 & 5). Dispice this the hemicellulose content of birch was substantially diminished whereas in spruce wood the cellulose losses were higher than that observed for hemicellulose. Wood-*Pi* removed only hemicellulose and lignin in both types of wood but cellulose remained almost undegraded. In contrast, degradation of these lignocellulose components was higher in birch compared to spruce indicating that hardwood hemicellulose was more easily degraded. However, no xylanase activities were found in solid state cultures. Furthermore in liquid medium using birch xylan as inducer for xylanases, negligible xylanase activity was only occasionally detected (unpublished). The absence xylanase activity in culture extracts suggested that the con-

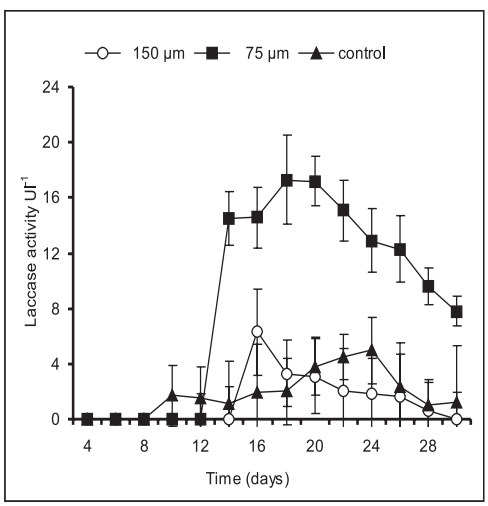

Figure 16 A. Production of laccase by *Paecilomyces inflatus* in liquid cultures *(Czapek-Dox)* supplemented with 75 µM (*black squares*) and 150 µM *(white circles)* copper sulphate *($CuSO_4$)*. *Black triangle* - control cultures without supplementation of $CuSO_4$. The experiment was performed at 28°C for 30 days. Data points represent means of three replicates (n = 3) with standard deviations *(bars)*.

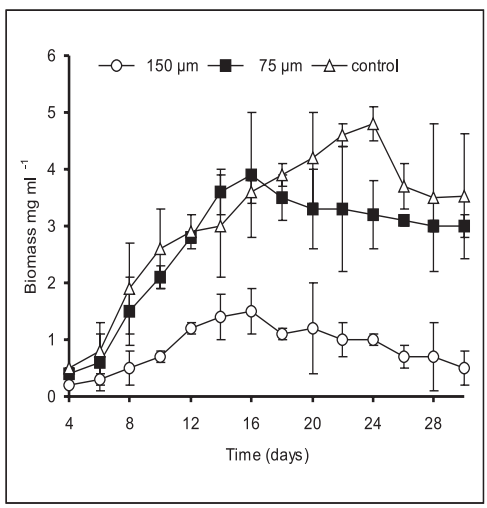

Figure 16 B. Biomass production by *Paecilomyces inflatus* in liquid cultures (Czapek-Dox) supplemented with 75 µM (*black squares*) and 150 µM (*white circles*) copper sulphate ($CuSO_4$). *White triangle* - control cultures without supplementation of $CuSO_4$. The experiment was performed at 28°C for 30 days. Data points represent means of three replicates (n = 3) with standard deviations *(bars)*.

ditions were unsuitable for extraction of these enzyme activities, which may have remained bound to the substrate.

During the experiment changes in pH were followed (IV). All fungal strains were found to adapt to ambient pH and degrade lignocellulose compounds from different waste materials to the same extent. The highest degradative capacities were found in that environment where the strain originally was isolated from. Thus, the compost-dwelling *P. inflatus* was the most effective (active) in compost whereas the Wood isolate was most effective in woody materials and Rhizo-*Pi* in straw material.

4.5. Cellulose-degrading endoglucanase (III and IV)

The predominant hydrolytic enzyme found in *P inflatus* strains Comp-*Pi* and Rhizo-*Pi* was endo-1,4-β-glucanase (EG). Surprisingly no EG activity was found in Wood-*Pi*. Comp-*Pi* produced most EG at the beginning of the experiment in straw and compost, whereas the production of EG by Rhizo-*Pi* was more apparent in wood cultures (IV, Figures 2a–d). In longer-term experiments the EG activities of Rhizo-*Pi* kept increasing up to 8 weeks.

EG production was studied in defined media amended with different supplements in order to characterize EG and its production by *P. inflatus* in more detail (III). The Czapek-Dox liquid medium was separately supplemented with glucose, cellobiose, CM-cellulose, Avicel cellulose, citrus pectin, meat peptone, yeast extract, ammonium salts, HA or veratric acid. The media containing CM-cellulose, cellobiose, citrus pectin and meat peptone significantly produced EG activity. Activity produced in the presence of CM-cellulose was nearly 10-fold greater than that produced in media containing glucose. Meat peptone supported the growth and high EG production compared with other nitrogen sources. *P. inflatus* was able to utilize nitrate and ammonium salts as pure nitrogen source in media containing cellulose.

Basal medium with HA and veratric acid increased EG production over controls(III, Figure 5) but had no effect on the mycelial dry weight of *P. inflatus* (III, Figure 4).

4.6. Modification of humic substances

A natural humic acid extracted from compost (CHA) and synthetic ^{14}C-labeled humic acid (^{14}C-HA) prepared from [U-^{14}C] catechol in compost and liquid cultures was modified by *P. inflatus*. Degradation resulted in the formation of low-molecular mass fulvic acid-like compounds (FAs) and carbon dioxide. Half of of HA (50 %) was polymerized into alkaline insoluble material (refractory humin; II, Fig.1).

Modification of the natural compost HA was easily detected by its partial decolourization in liquid culture (II, Fig. 5 A, B). Bleaching of the medium was accompanied by moderate changes in the molecular mass distribution of both HA and FA fractions (II, Fig.5A &B). HA modification was most pronounced during the primary growth phase of the fungi and was associated with increased laccase activity. The low concentration of HA (250 mg ml^{-1}) in culture medium increased fungal laccase and EG activities significantly (II, Fig. 3; III, Fig. 5).

5. DISCUSSION

5.1. Degradation of lignin (I and IV)

Paecilomyces inflatus strains were capable of decomposing the lignin-cellulose complex from composting waste materials. The production of hydrolytic and oxidative enzymes was accompanied by the loss of total mass and the lignocellulose content. It was regulated by temperature and pH (I, IV).

Paecilomyces inflatus was found to be able to mineralize side-chain $^{14}C_{\beta}$-labeled synthetic lignin (DHP) to a moderate extent. *P. inflatus* strain BKT02 proved to be the most active *P. inflatus* strain investigated and converted 0.9 % of the labeled lignin per week into $^{14}CO_2$ and 10 % within 12 weeks. The wood–rotting ascomycete *Xylaria polymorpha* mineralizes similar amounts of the same type of DHP in birch wood cultures during a comparable time to that used in our experiment (Liers et al. 2006). The production of $^{14}CO_2$ by *P. inflatus* was growth-associated, suggesting that the attack on lignin occurred in the primary phase of fungal metabolism, which agrees with the results of Falcon et al. (1995) and Regalado et al. (1997). This finding is in contrast to the white rot fungi in which extracellular enzymatic system and lignin degradation are typically secondary metabolic events (Keyser et al. 1978, Kirk and Farrell 1987).

Apart from its ability to mineralize *P. inflatus* also depolymerized ^{14}C-labeled lignin resulting in the formation of ^{14}C-labeled water-soluble lignin fragments. The water-soluble fraction includes low-molecular weight lignin fragments (intermediates), which can be either taken up and further catabolised by the fungal hyphae of white rot or be released as indigestible fragments resistant to further biodegradation. The amounts of ^{14}C solubilized in the compost medium during the growth of *P. inflatus* strains were approximately 1.5 fold that of $^{14}CO_2$ evolved. In this context, *P. inflatus* resembles certain wood-rotting ascomycetes and litter decomposing basidiomycetes i.e., *Stropharia semiglobata* (Steffen et al. 2000, Liers et al. 2006) as well as certain strains of bacteria (*Streptomyces;* Berrocal et al. 1997). In contrast, studies on basidiomycetes have shown that for certain of these fungal species such as *Nematoloma frowardii*, rates of the mineralization are approximately tenfold of solubilization rates of ^{14}C-labeled lignin (Hofrichter et al. 1999). This suggests different mechanisms of substrate attack among different lignin degrading microorganisms. For example, mineralization being more pronounced in white rot basidiomycetes.

In SSC, most of the radioactivity was found in the water-insoluble fraction, which suggests that a substantial portion of the ^{14}C-labeled lignin was polymerized into more recalcitrant high molecular weight compounds (Tuomela et al. 2001). These compounds were accumulated and possibly stabilized by covalent linkages to humic substances (Shevchenko and Bailey 1996, Almendros et al. 2000, Tuomela et al. 2001). Unlike *P. inflatus*, white rot fungus *Trametes versicolor* was capable of degrading and mineralizing humic-bound lignin, as it constantly produces water-soluble degradation products from humic-bound lignin during incubation in soil (Tuomela et al. 2002).

Lignin degradation capability of microfungi has been evaluated with radiolabeled compounds in only a few studies(Haider and Trojanowski 1975, Falcon et al. 1995, Re-

galado et al. 1997, see introduction heading 1.2.2.2). Haider and Trojanowski (1980) used specifically methoxyl-C, side chain-C or ring-C labeled lignins as substrates observed higher mineralization of side chain-C and methoxyl-C labeled lignin compared to that of ring-C-labeled lignin preparations. Thus the mineralization of side-chain $^{14}C_{\beta}$-labeled-lignin was about 10 % compared with less than 4 % mineralization of ^{14}C-(ring)-labeled DHP (Falcon et al. 1995, Regalado et al. 1997, Anderson et al.2005). Furthermore, Rodriguez et al. (1996b) found a maximum of 20-27 % conversion of ^{14}C-labeled lignin from wheat straw to $^{14}CO_2$ in 28 days. Lignin prepared from pine wood was much less degraded and about 3 % of ^{14}C-labeled lignin was mineralized. This was attributed to the intrinsic difference between the two lignin types (Rodriguez et al. 1996b, Regalado et al. 1997).

The decrease in the Klason content after growth with *P. inflatus* BKT 02 in compost confirmed the capability of this fungus to delignify lignocellulose residues. *P.inflatus* degraded lignin simultaneously with the carbohydrate fraction (cellulose and hemicellulose). The degradation mechanisms of *P. inflatus* in compost resembled those described for soil-inhabiting microfungi such as *Fusarium solani, F. oxysporum* and *Penicillium chrysogenum* (Rodriguez et al. 1996b). Losses of cellulose and hemicellulose in compost inoculated with *P. inflatus* were high over the initial period whereas lignin decomposition was more extensive at a later stage (IV). In fact, the large lignin loss became detectable after fungal biomass stopped accumulating and the formation of soluble sugars due to hydrolytic activities decreased. Losses of lignin during solid-state fermentation in compost by *P. inflatus* were similar with those obtained in *Fusarium oxysporum, Penicillium chrysogenum* and *Monilia sterilia* growing on wheat and oat straw (Rodriguez et al. 1996b, Stepanova et al. 2003).

The treatment of compost with *P. inflatus* BKT 02 to some extent changed the pattern of molecular mass distribution of lignocellulose within a period of 8 weeks. Fungus primarily released water-soluble lignocellulose fragments of medium (25–30 kDa) and small size (0.6 kDa) In contrast, the ascomycete *Xylaria polymorpha* forms high amounts of water-soluble lignocellulose fragments of larger size (~30 kDa) during growth on beech wood (Liers et al. 2006); a fact that was attributed to the action of an esterase cleaving the bonds between hemicelluloses and lignin. Hofrichter et al. (2001) have reported that the white rot basidiomycete *Phlebia radiata* MnP preferentially releases small lignocellulose fragments of pine wood. This data may indicate that peroxidase activities present in white-rot fungi are responsible for degradation of lignin into small fragments.

Unlike compost, *P. inflatus* was found to be capable of causing substantial lignin mass loss in other lignocellulosic materials such as wheat straw and wood. Lignin removal or modification during degradation of these substrates varied between the *P. inflatus* strains that colonized the same plant material. Thus, they showed different responses to lignocellulosic materials in addition to varying cultivation conditions. Cellulose and hemicellulose were removed first in wood and straw followed by lignin decrease indicating different strategies of lignocellulose degradation in wood and in straw to that of compost. In general, the extent of lignin degradation in compost was higher than in straw or woody materials. Softwood and hardwood lignin was degraded approximately to the same degree by two strains of *P. inflatus* originating from habitats containing woody ma-

terials. They degraded lignin at least as efficiently as the xylariaceous ascomycetes that are known to cause white rot-like decay and also as efficiently as some marine and freshwater fungi (Sutherland et al. 1982, Worrall et al. 1997, Bucher et al. 2003, Pointing et al. 2003). Decay of wood by P. *inflatus* occurred at acidic pHs similar to that for like white rot fungi, whereas lignin from compost and straw was preferentially degraded at slightly alkaline pHs.

Interestingly, the coprophilous mushroom *Coprinus radians* does not produce ligninolytic peroxidases (LiP, MnP or VP) at low pH (2.5–5.5) instead in more alkaline environments has developed alternative strategies for transforming aromatic substances, using peroxygenases (Anh et al. 2007). Compost is characterized by relatively high amounts of nitrogen and high pHs (pH 6–9), which provides circumstantial evidence for the assumption that the production of peroxygenases may be a characteristic feature of alkaliphilic fungi such *P. inflatus*. Peroxygenases were not investigated in this study.

In many cases, nutrient content in cultivation medium has been shown to be very important for lignin degradation rates and production of specific enzymes. Thus, nutritional regulation may be also relevant to the natural habitat of *P. inflatus* strains. Low N conditions, which normally pertain in woody materials, enhance lignin degradation in *Phanerochaetae chrysosporium* (Fenn and Kirk 1981) whereas low carbon and low nitrogen conditions in soil are both prerequisite for *Fusarium proliferatum* lignin degrading activity (Anderson et al. 2005).

To summarize, the results of the lignin degradation study indicate that *P. inflatus* was able to degrade lignin in a compost and in other lignocellulosic materials. The reason for the less efficient degradation may be the lack of ligninolytic peroxidases in *P. inflatus*, the production of which seems to be a specific feature of white rot fungi. Thus, fungi, which produce only one of the ligninolytic enzymes (mostly laccase) or do not produce any ligninolytic enzymes at all may reach only low levels of lignin degradation/mineralization, compared to those species producing both peroxidases and laccase (Steffen et al. 2000).

5.2. Lignin-degrading laccase

P. inflatus exhibited highest laccase activity at neutral to slightly alkaline pH values (6.5 to 7.5), which resembles laccase production of certain compost fungi such as *Chaetomium thermophilum* (Chefetz et al. 1998b) and also laccases directly isolated from compost (Chefetz et al.1998a). This production pattern differs from that of many white rot fungi, which usually form substantial amounts of organic (carboxylic) acids (especially oxalic acid) and acidify the medium during lignin degradation. Typically laccases produced by white rot fungi are the most active in acidic pHs, whereas laccase in *P. inflatus* as shown in this study and in other microfungi acting at neutral pH (Chefetz et al. 1998, Stepanova et al. 2003). *Paecilomyces* spp. usually grow at slightly alkaline environments (pH 7–9) (Magan 1997), which explains the divergent behaviour concerning laccase production and pH optima.

In many fungi, the ligninolytic enzyme system is switched on in response to nutrient starvation (Collins and Dobson 1997, Gianfreda et al. 1999). A considerable amount of *P. inflatus* laccase was found in high-nitrogen cultures containing peptone. Peptone from meat was also found to be the most effective nitrogen source for the ascomycete *Penicil-*

lium simplicissimum (Zeng et al. 2006) and the basidiomycete *Trametes pubescens* (Galhaup et al. 2002). Unfortunately molecular analyses of laccase expression in ascomycetes has not yet been published, so it is not known, if at least some of the increased activity is attributed to the higher biomass formation. Studies on the effect of N concentration on fungal laccase genes at the transcriptional level has been studied in several basidiomycetes fungi such as *Trametes versicolor and Pleurotus sajor-caju* (D´Souza et al. 1996, Collins and Dobson 1997, Mansur et al. 1998, Soden and Dobson 2001). These studies demonstrated that under high nitrogen (10mM ammonium tartrate) culture conditions laccase transcript levels increased as much as 100-fold compared to transcript levels under limited N conditions in basidiomycete I-62 (Mansur et al. 1998).

High nitrogen media give the highest laccase activity in the ascomycete *Penicillium simplicissimum* (Zeng et al. 2006) and the mitosporic fungi *Pestalotiopsis* sp. (Hao et al. 2007) and *Monotospora* sp. (Wang et al. 2006) whereas the nitrogen-limited conditions enhance laccase production in the basidiomycetes *Pycnoporus cinnabarinus* (Eggert et al. 1996), *Phlebia radiata* (Gianfreda et al. 1999), *Trametes pubescens* (Galhaup et al. 2002) and in the freshwater fungi *Dactyllela submersa* and *Flagellospora penicillioides* (Abdel-Raheem 1997).

Nitrate salts were found to be a better nitrogen source than ammonium salts in *P. inflatus,* in contrast to findings obtained by Wang et al. (2006) and Hao et al. (2007), who studied mitosporic fungi *Pestalotiopsis* sp. and *Monotospora* sp. Both group of authors observed that ammonium tartrate was beneficial for laccase production in these species. When ammonium salts in the form of sulphate or phosphate were used as the nitrogen source in *P. inflatus* cultures, the pH of the medium always decreased between 4.7 and 5.0 during cultivation, whereas with nitrate and organic nitrogen sources, the pH rose to over 7.0 (Table 9).This finding correlates well with the preferential alkaline pH regime for the growth of *P. inflatus*.

The type of the carbon source in the medium also affected laccase production in *P. inflatus*. Xylan gave the highest laccase activity but cellobiose and xylose were also good carbon sources for laccase production (Table 9). Starch, pectin and CM-cellulose supported excellent mycelium growth, but laccase activity remained at a low level. Xylan has been reported to induce laccase formation in the ascomycete *Botryosphaeria* sp., but xylose, a product of xylan hydrolysis by fungal xylanase was shown to function as a laccase inducer (Dekker et al. 2001). When using glucose as the substrate, laccase activity increased after glucose was depleted from medium. This finding is most likely due to the glucose repression effect, which is proposed to be an energy-saving response of fungi and yeasts (Ronne 1995). Interestingly, low concentration of glucose in *Fusarium proliferatum* promoted early production of both mycelial and extracellular laccases (Kwon and Anderson 2001, Anderson et al. 2005).

In solid-state cultures *P. inflatus* BKT 02 produced the highest laccase amounts in compost medium. Over two times more laccase activity was found in compost compared to straw and wood. These results obtained under SSC conditions are consistent with the results obtained in liquid cultures. Moreover, they showed that when supplemented with compost extracts, laccase production increased up to two-fold followed by wheat straw and spruce wood extracts supplementation of which gave four–and threefold higher enzyme titers. The fact that *P. inflatus* had higher laccase activity in solid state and liquid cultures containing lignocellulose than in media with high amounts of glu-

cose may be explained by the presence of inducing compounds such as lignins, phenols and other aromatics such as fulvic acids in compost originating from plant materials. In some other fungi such as *Trametes versicolor,* the presence of lignocellulosic residues significantly stimulated laccase production in culture medium (Lorenzo et al. 2002). Soybean meal as both a carbon and nitrogen source in *Xylaria polymorpha* resulted in much higher yield of laccase than in mineral medium (Liers et al. 2006). Furthermore, wheat straw in *Thermoascus aurantiacus* (Machuca et al. 1998), lignosulfonate in *Botryosphaeria* sp. (Dekker et al. 2002), cotton stalk extract in *Pleurotus ostreatus* (Ardon et al. 1996), cereal bran extracts in *Coriolopsis gallica* UAHM 8260 and *Bjerkandera adusta* UAMH 8258 (Pickard et al. 1999), and pectin in *Botrytis cinerea* (Marbach et al. 1985) have yielded significant quantities of laccase.

In SSC *P. inflatus* strains gave different results with respect to growth on lignocellulosic materials and to laccase production. Strain Wood-*Pi* produced the highest levels of laccase in birch and spruce after 12 weeks of cultivation, whereas laccase production by the strain Comp-*Pi* in straw and composting material occurred earlier and reached its maximum level within 8 weeks. Although the compost medium did not stimulate laccase activity significantly in other strains, Wood-*Pi* degraded lignin to the same extent as Comp-*Pi* did. The maximal activity of laccase did not directly correspond to the timing of the maximum lignin degradation in all SSC. It is possible that laccase activity in aromatic-rich compost media may not be solely connected with lignin degradation but may preferentially promote the polymerization and/or detoxification of phenolic/aromatic compounds, a well-known feature of laccases (Baldrian 2006). On the other hand, only low levels of laccase activity were needed for the ligninolytic system of *Petriellidium fusoideum*, where the activity was correlated with production of hydroxyl radicals (Gonzales et al. 2002). It should be pointed out that hydroxyl radicals were not investigated in this study.

Vanillic and vanillic acid were found to be most effective, in laccase production by *P. inflatus* resulting in a 4-fold increase in laccase production over the uninduced control (I, Figure 5) whereas veratryl alcohol and veratric acid only slightly induced laccase activity. This is in contrast to the results of studies on *Botryosphaeria* sp. and *Coniochaeta* sp where veratryl alcohol extensively induced laccase production and where the enzyme levels were dependent on the concentration of the inducer used (Barbosa et al. 1996). All aromatic compounds used in this study lead to diminished fungal biomass production. It has been proposed that fungal laccases during the polymerization of toxic aromatic compounds function as a defence mechanism against oxidative stress (Eggert et al. 1996, Fernandez-Larrea et al. 1996). We suggest it could also occur in the case of *P. inflatus* too.

Many aromatic compounds such as xylidine, veratric acid, veratryl alcohol, vanillin, vanillic acid, and metal ions can elevate laccase production levels (Eggert et al. 1996, Collins and Dobson et al. 1997, Regalado et al. 1999, Gianfreda et al. 1999, Junghanns et al. 2005). However, in ascomycetes neither laccase activity nor lignin mineralization rates were enhanced by xylidine (Bollag and Leonowicz 1984, Rodriguez et al. 1996a, Machuca et al. 1998, Liers et al. 2006).

The addition of copper as $CuSO_4$ seems to be beneficial for the increased of laccase production in many fungi (Palmieri et al. 2000, Galhaup et al. 2001). Copper has a posi-

tive effect on laccase stability (Baldrian and Gabriel 2002) and can mediate the inhibition of an extracellular protease that degrades the laccase proteins (Palmieri et al. 2001). Induction of laccase synthesis by copper is widespread among fungi. The induction mostly acts on the gene transcription level as has been shown for *Hortaea acidophila* (Tetsh et al. 2005), *Gaeumannomyces graminis* (Litvintseva et al. 2002), *Podospora anserina* (Fernandez-Larrea and Stahl 1996), *Pleurotus ostreatus* (Palmieri et al.2001) and different *Trametes* spp. (Collins and Dobson 1997, Galhaup et al. 2001).

The optimal concentration of copper for *P. inflatus* was found to be 75 μM, leading to a 4-fold increase of laccase production when compared with control without copper. Higher concentrations of $CuSO_4$ (up to 150 μM) were detrimental for the fungus. So copper was not very efficient laccase inducer in *P. inflatus*. Some other fungi are also quite sensitive to copper, such as *Trametes pubescens* (Galhaup et al. 2001), whereas others appear to tolerate high amounts of copper (2 mM; D´Souza et al. 2004).

5.3. Degradation of cellulose and hemicellulose (IV)

The decomposition of cellulose also varied considerably in *P. inflatus*, accounting for the loss of 40 to 60 % of the total dry mass of birch and spruce, 14–35 % of compost and 11 to 20 % of straw. Losses of cellulose from the lignocellulosic substrates were higher than that of lignin or hemicellulose alone. The highest cellulolytic activities were evident from the first weeks of incubation with P. *inflatus* strains. These resulted in rapid cellulose degradation and also high levels of released sugars. It is interesting that in contrast to other strains Wood-*Pi* had a very low capacites to degrade cellulose under the conditions set. The ability of *Paecilomyces* spp. to degrade cellulose has been reported elsewhere (Eslyn et al. 1975, del Rio et al. 2001, Martinez et al. 2005), which show the variability of the genus *Paecilomyces*. The experimental data suggest that cellulose degradation by the strain Wood-*Pi* was possibly regulated by other mechanisms than those of strains that readily produce cellulolytic enzymes. However, the mechanism was not investigated in detail in this study.

The rate of the decomposition of hemicelluloses, in all lignocellulolytic materials, was almost constant throughout the incubation period, although Rhizo-*Pi* depleted more hemicelluloses from straw and hardwood than the other strains. Strain Rhizo-*Pi* exhibited a preference for xylan, the main component of hemicelluloses in hardwood and grasses. The hemicellulose fraction of birch was substantially reduced in weight by all the strains as early as four weeks after the start of incubation. The action of the fungus may have resulted in the low pH values observed in birch wood. Fungal xylan degradation can result in the formation of acetate, glucuronic acid and ferulic acid (Perez et al. 2002), which, in turn, may lower the pH in the decayed wood. However, even though the hemicellulose fraction was clearly diminished in birch, xylanolytic activity for *P. inflatus* was not detected.

All strains of *P. inflatus*, regardless of their origin, altered the ambient pH in a similar manner in all tested substrates (IV). The neutral or slightly alkaline optimum pH for growth of *P. inflatus* would suggest that the lignocellulose degradation should also operate under similar conditions. However, the highest enzymatic activities produced by *P. inflatus* strains as well as degradation rates of all lignocellulose components were found at alkaline pHs only in compost and straw, whereas they were found at acidic pH in birch

and spruce. In nature, many extracellular enzymes including xylanases, cellulases or laccases are under a pH regulated system, which ensures that these enzymes are produced under conditions of optimal pH (Denison et al. 2000, Peñalva and Arst 2002). This phenomenon has been shown to operate in some ascomycete fungi and yeasts (Denison 2000, Peñalva and Arst 2002). A similar regulatory system may also operate in *P. inflatus*, which may result in differential production of wood-degrading enzymes in different ambient pH values. This could explain the lack of xylanolytic activity at acidic medium of *P. inflatus*. These findings suggest that all *P. inflatus* isolates may regulate the pH in their micro-habitat.

According to the literature, the decomposition process of ascomycete microfungi involved the enzyme mediated decay of polysaccharides accompanied by little or no lignin degradation (Deacon 1997). The present study revealed two different types of lignocellulose degradation by *P. inflatus* fungi. In the preferential type (i) cellulose and hemicellulose were removed first followed by lignin removal, whereas in the simultaneous type (ii) all cell wall components were degraded concomitantly. The mode of the lignocellulose degradation in compost by *P. inflatus* BKT 02 resembled the second type (ii) of decay, which previously has been observed in only a few ascomycetes such as *F. solani, Oidiodedron maius* and *Acremonium* cf. *curvulum* (Rodriguez et al. 1996, Tsuneda et al. 2001). However, the degradation of lignocellulose by these fungi was restricted to the areas adjacent to or in direct contact with hyphae (Tsuneda et al. 2001).

5.4. Cellulose-degrading endoglucanase (EG; III and IV)

P. inflatus was able to produce endoglucanase (EG) in solid state cultures on complex lignocellulose substrates as well as in several liquid media. EG activity was significantly stimulated by certain lignin-related aromatic compounds. Repression of various cellulases by phenols has been previously demonstrated in *Chaetomium globosum*, *Schizophyllum commune* (Varadi 1972), *Botryosphaeria* sp. (Dekker *et al.* 2001) and in the anaerobic fungus *Baselophus tragocamelus* (Paul et al. 2003). Nevertheless, there is evidence that only very low concentrations of aromatic substances can slightly increase cellulolytic enzymes activity in a few white- and brown-rotting fungi (Müller et al.1988, Highley and Micales 1990, Tsujiyama 2003).Veratryl alcohol slightly enhanced EG production in *P. inflatus* whereas it inhibited EG activity in the ascomycete, *Botryosphaeria* sp. (Dekker *et al.* 2001).

Soil humic acid (SHA) and veratric acid were the most efficient elicitors of the cellulolytic activity in *P. inflatus*. Even low concentration of both compounds (250 mg l^{-1}) significantly enhanced EG titres, but had no effect on the fungal biomass. This increase in activity may be related to similar interaction of humic compounds with cellulases in soil as has been reported by Busto et al. (1997).

EG was the major cellulolytic enzyme produced by *P.inflatus* in solid state cultures of compost, straw and wood. Strain Wood-*Pi* did not produce measurable EG activities although cellulose was converted in compost and straw media. The apparent lack of EG activity may have been attributed to the adsorption of the EG onto the substrate and / or onto the fungal mycelia. Moreover, lignin present in lignocellulose complex may also act as an adsorbent for cellulase thus diminishing degradation of cellulose (Béguin and Aubert 1994).

Comp-Pi and Rhizo-Pi exhibited moderate enzyme activities whereas Wood-*Pi* showed no measurable EG activities in compost and straw. Even so, it could degrade the cellulose of both substrates to some extent. Maximal EG activities of the cellulolytic *P. inflatus* strains corresponded to the maximal rates of cellulose consumption in all tested materials. Some of the lignocellulosic substrates yielded very low activities (birch wood, wheat and oat bran), whereas the highest EG activities were obtained with compost, wheat straw and spruce wood. The highest EG levels in compost were detected at near neutral pH values (6.6–7.1) at 28°C. The EG activities notably dropped at higher pH values. Straw, which is used as medium for the efficient EG production in other fungi including *Aspergillus niger, Hypocrea jecorina* and *Neurospora crassa* (Romero et al. 1999, Thygesen et al. 2003) was also a good source for cellulase activity in *P. inflatus*. The fact that straw exhibited high EG activities as compared to other lignocellulolosic substrates may be related to their composition. Grass clippings are one of the substrates with a low lignin content in contrast to woody materials, where the lignin content is much higher. In wood tissues cellulose is surrounded by lignin, which may lead to a diminished degradation of cellulose (Béguin and Aubert 1994). A slightly alkaline pH in *P. inflatus* straw cultures was optimum, which agrees with results obtained in other studies (Romero et al. 1999, Ögel et al. 2001). The increase in pH observed in solid cultures containing lignocellulose may be due to fungal cell lysis or constituents of different enzymes secreted to utilize the substrate. This is in contrast to CM-cellulose amended liquid cultures, in which no EG activity was observed at alkaline pH values but only a narrow pH optimum (5.0–6.5) of EG production by *P. inflatus* were obtained. These changes in pH in liquid and solid state cultures may suggest differential EG production depending on liquid or solid-state culture conditions as well as a nature of the substrate used. In liquid cultures supplemented with various carbon sources an increase in pH was observed whereas the pH in pectin containing cultures decreased notably, presumably due to the fomation of galacturonate moieties (Dutton and Evans 1996, Green III et al. 1996). It is important to mention that *P.inflatus* posses some pectinolytic activities and produced oxalic acid when grown on pectin (C. Sivelä, personal communication).

Amorphous celluloses and cellobiose stimulated the production of cellulase by *P. inflatus*. The moderate EG activities were associated with the low expression of *β*-glucosidase. The low *β*-glucosidase activities detected in liquid cultures were possibly associated with a partial repression of the enzyme by glucose released during the induction by cellobiose. Growth in the presence of cellobiose, a product of cellulose hydrolysis has been shown to induce cellulase expression in many species of fungi including all the main cellulases of *Hypocrea jecorina* (= *Trichoderma reesei*; Ilmén et al. 1997), EG of *Aspergillus nidulans* (Chikamatsu et al. 1999), EG of *Mucor circinelloides* (Saha 2004) and *β*-glucosidase of *Aspergillus terreus* (Pulshalkar et al. 1995). However, the reports concerning the inducing effect of cellobiose have been somewhat controversial. This is most likely due to the varing culture conditions and cellobiose concentrations used in laboratory experiments (Aro et al. 2005).

In addition to the carbon source, other factors such nitrogen source is important for EG production. Production of EG by *P. inflatus* was much higher than that for inorganic nitrate and ammonium salts when complex nitrogen sources (peptone, yeast extract) were used. Peptone appeared to be a superior source of nitrogen for *P. inflatus* growth,

and also for EG production. This is similar finding to that of the ascomycete *Neurospora crassa,* the fungus closely related to the *Paecilomyces inflatus* (Yazdi *et al.* 1990, Luangsa-ard et al. 2004). Organic nitrogen may enhance the production of fungal proteases, which are capable activating EG activity in white rot fungus *Phanerochaete chrysosporium* (Eriksson and Pettersson 1982). In contrast, the production of *β*-glucosidase by *P. inflatus* was higher when nitrate rather than peptone was used, which is in accordance with data presented for *Aspergillus terreus* (Pulshalkar et al. 1995).

5.5. Modification of humic substances (II)

The compost-dwelling ascomycete *P. inflatus* decomposed natural HAs and synthetic HAs in liquid and compost solid state cultures. The degradation resulted in the formation of lower molecular mass FA-like compounds and carbon dioxide. As in the case of lignin degradation, HA modification in compost was most pronounced during the primary growth phase of *P. inflatus*. Furthermore, the major fraction of ^{14}C-HA (50 %) was polymerized into alkaline insoluble material. This finding indicates that *P. inflatus* was not only able to degrade HA but also to further polymerize HS resulting in the formation of refractory humic substances known as humin. In the study by Tuomela et al. (2001), 39 % of the applied ^{14}C-labeled lignin was found to be bound to the humin fraction at the end of the composting experiment at 6 weeks. This phenomenon has also been observed in cultures of litter-decomposing fungi in sterilized litter, where most of the ^{14}C-DHP (60 %) remained bound to humus (K. Steffen, personal communication).

In most cases degradation of HA occurs co-metabolically and with easy assimilate carbohydrates often serving as the carbon source (Gramss et al. 1999). However, no significant effect of carbohydrate (glucose) supplementation was observed on the conversion of HA by *P. inflatus*. The bleaching (decolourization) of dark-brown compost HA was more prominent in liquid culture media containing HA as the sole carbon source compared with cultures using HA supplemented by glucose. Moreover, the growth of the fungal mycelia was clearly stimulated in the presence of HA (II). These results are in agreement with those of Řezáčová et al. (2006) who reported that the common microfungal species *Clonostachys rosea* and *Paecilomyces lilacinus* were able to grow on soil HA and decolourize them in addition modifying soil HA chemically. The decolourizing capability of HA by another microfungus *Chalara longipes* (Koukol et al. 2004) was even higher than that previously found for basidiomycetes *Coriolus consors*, *Coriolus hirsutus* and *Lenzites betulina* (Yanagi et al. 2002).

P. inflatus caused a modest decrease in the relative concentration of HA in Czapek-Dox medium. This correlated with the extent of decolourization. Simultaneously the amount of FA increased indicating partial oxidation of the HA material as suggested by William and Fakoussa (1997b). HPSEC analysis reveald a greater decrease in molecular mass of HA than those reported for some other microfungi (Hofrichter et al. 1997, Gramss et al. 1999).

Studies of HA decolourization, depolymerization or mineralization have mainly focused on a few model species of the ligninolytic white rot fungi such as *Phanerochaete chrysosporium, Trametes versicolor* or *Nematoloma frowardii* (Blondeau 1989, Dehorter and Blondeau 1992, Hofrichter et al. 1998b)and also in fungi that are not able survive in compost or soil for a prolonged time (Dix and Webster 1995). However, the release

of $^{14}CO_2$ from ^{14}C-HA and decolorization of HA under co-metabolic conditions was shown to occur in cultures of the litter decomposing mushroom *Collybia dryophila* (Steffen et al. 2002). Those authors demonstrated that the ability of litter-decomposing basidiomycetes to modify HS is associated with their extracellular enzyme system comprising non-specific ligninolytic enzymes. It is assumed that peroxidases (MnP and LiP) are involved in the transformation of humic substances (Blondeau 1989, Dehorter and Blondeau 1992, William and Fakoussa 1997a, Hofrichter et al. 1998, Steffen et al. 2002) although laccase is also most probably involved (Zavarzina et al. 2004). Laccases are also produced by wood- and litter-decomposing mushrooms in addition to microfungi (Thurston 1994, Steffen et al. 2000, Hatakka 2001), *Streptomyces* (Berrocal et al. 2000) and even other bacteria (Martins et al.2002).Laccase activities can be found in the upper soil horizons, litter samples, and in compost (Chefetz et al. 1998b, Criquet et al. 1999, Gramss et al. 1999) indicating laccase involvement in the humus formation and turnover. More evidence for this hypothesis was later provided by Zavarzina et al. (2004), who demonstrated that laccase of the basidiomycete *Panus tigrinus* was responsible for both polymerization and depolymerization of soil and peat-derived HA.

Since peroxidases are produced mainly by basidiomycetes (Hofrichter 2002), other enzymes must be responsible for the HA conversion by *P. inflatus*. The fungus produced only laccase in liquid cultures supplemented with compost HA and its laccase activity was increased in the presence of HA (II, Figure 3). The stimulation effect of HA on laccase activity has been observed in several basidiomycete fungi (Temp et al. 1999, Scheel et al. 2000). Moreover, the maximum level of laccase activity in *P. inflatus* coincited with the decolourization of high molecular mass HA and their conversion into low-molecular mass FA (II). Similar observations were reported by Claus and Filip (1998) as well as Fakoussa and Frost (1999) for the decolorization of HA and their depolymerisation to FA by *Cladosporium cladosporioides* and *Trametes versicolor*. In both cases, high activities of laccase are detected.

Compost-colonizing microfungus *P. inflatus* with ligninolytic activities were observed to be involved in compost HA transformation and thus may play an important role in humus formation and turnover in a composting environment.

6. CONCLUSIONS AND FUTURE PERSPECTIVES

The anamorph strains of an ascomycete *P. inflatus* isolated from compost were capable of decomposing natural lignin from compost and synthetic $^{14}C_{\beta}$-labeled lignin (^{14}C-DHP) prepared from coniferyl alcohol. Evidence for the ligninolytic activity of *P. inflatus* was found in the lignin mineralization experiments. *P. inflatus* strains mineralized 6–10 % of radiolabeled lignin to $^{14}CO_2$ within 12 weeks of the incubation in a compost environment. Apart from the mineralization fungi also depolymerized ^{14}C-labeled lignin resulting in the formation of ^{14}C-labeled water-soluble lignin fragments. However, most of the lignin was not mineralized but bound to the insoluble humin-like fraction as expected, since lignin is the major precursor of all humic substances.

The chemical analysis of the compost after 12 weeks of growth and treatment with *P. inflatus* BKT 02 also revealed that the fungus was capable of degrading lignin simultaneously with the carbohydrate fraction. HPSEC analyses showed that *P. inflatus* in compost released a small amount of water-soluble lignocellulose fragments of larger size whereas the amount of medium- and small fragments were moderately increased. This may indicate some modifications in lignocellulose fibres, which may be attributed to lignocellulose-degrading enzymes produced during P. *inflatus* growth in the compost. However, the role of these enzymes is still not clear. The hypothetical degradation mechanism of ascomycetes including *P. inflatus* suggested by results obtained in this and previous studies is presented in Figure 17**.**

P. inflatus converted a synthetic labeled humic acid (^{14}C-HA) prepared from [U-^{14}C] catechol and humic acids (HAs) extracted from authentic compost. Mineralization experiments with ^{14}C-labeled humic acids revealed that *P. inflatus* was also capable of degrading HA in addition to stimulating the formation of refractory humins. As the result of fungal enzymatic activity high molecular mass HAs were converted to smaller fulvic acids and $^{14}CO_2$. The degradation of HS, HAs and also modified lignin fragments suggests an important role of *Paecilomyces* spp. in humus turnover.

Laccase was the only oxidoreductase identified in *P. inflatus*. The production of the enzyme was correlated with mycelial growth. Laccase was stimulated in the presence of aromatic and lignin related compounds. The presence of natural HA in liquid cultures noticeably induced laccase production, which leads to the conclusion that laccase, may be directly involved in HA degradation. Laccase had the highest activities at neutral pH, suggesting the important role of this enzyme in composting and humification.

Decay of compost cellulose was moderate and showed preference for amorphous cellulose. Thus *P. inflatus* expressed noticeable amounts of enzymes cleaving cellulose (endoglucanase and occasionally β-glucosidase). EG of *Paecilomyces* spp. seemed to be involved in the degradation and transformation of residual cellulose moieties in compost because its activity was found to be associated with the decrease in cellulose content in compost.

Degradation of different plant materials and production of lignocellulytic enzymes may indicate flexible adaptation strategies in *P. inflatus*. The ability of *P. inflatus* to grow, to secrete laccase and to degrade lignin in addition to producing endoglucanase over varying pH and temperature ranges in the presence of phenolics and organic nitrogen

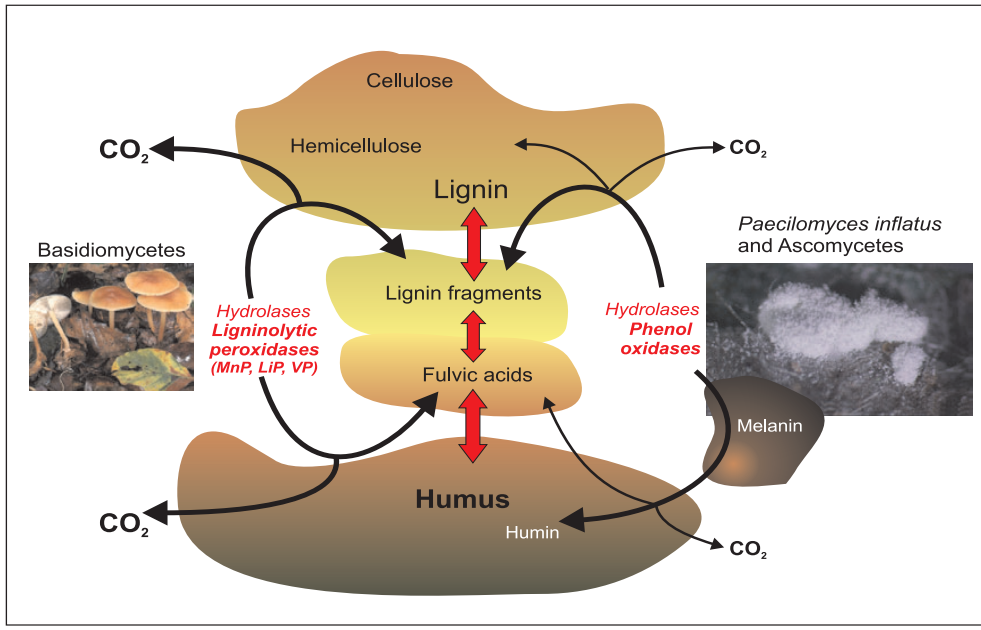

Figure 17. Hypothetical lignocellulose degradation mechanisms of P. inflatus in compost in the light of results obtained in the studies discussed in this disseratation and previous literature after Hofrichter et al. (2005) with modifications.

sources indicates that this fungus is well adapted to degrade solid plant materials in harsh compost conditions. The degradative features of this species of microfungi are of general relevance for lignocellulose decomposition in nature, especially in soil and compost environments, where basidiomycetes are not established at all or only poorly so. In conclusion, an important role of *P. inflatus* and related microfungi in carbon recycling can be expected in natural habitats.

Although our study showed that *P. inflatus* can degrade lignocellulose complex in compost with particular respect to recalcitrant lignin, there are few aspects that must be further studied. First, the capability of the fungi to compete with the normal microbial population of compost should be investigated. This includs studing and determinating the interactions with other compost-dwelling fungi in order to understand the role of compost-dwelling fungi in the carbon transfer during the composting process. Second, further investigations are needed to examine the degradative activities of compost microfungi including *P. inflatus* in particular the oxidative enzymes that are involved. Moreover, the role and properties of the enzymes should be clarified in more detail to widen our understanding of their significance for the composting and humification processes.

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